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(54) **Title:** EXPRESSION SYSTEMS FOR INSECT PEST CONTROL

(57) **Abstract:** Promoters active in insects can be enhanced by positive feedback mechanisms and associated with repressible lethal effects.

EXPRESSION SYSTEMS FOR INSECT PEST CONTROL

The present invention relates to insect expression systems comprising a promoter.

The genetic manipulation of insect species other than *Drosophila melanogaster*, by recombinant DNA methods, is in its infancy (Alphey, 2002; Alphey and Andreasen, 2002; Alphey *et al.*, 2002; Benedict and Robinson, 2003; Berghammer *et al.*, 1999; Catteruccia *et al.*, 2000; Coates *et al.*, 1998; Handler, 2002; Horn *et al.*, 2002; Jasinskiene *et al.*, 1998; Lobo *et al.*, 2002; Lozovsky *et al.*, 2002; McCombs and Saul, 1995; Moreira *et al.*, 2004; Peloquin *et al.*, 2000; Perera *et al.*, 2002; Scott *et al.*, 2004), and very few transgenic lines of non-*Drosophila* insects have been made, using heterologous promoters.

Insect transformation is a low-efficiency system requiring the identification of rare transformants, in a background of larger numbers of non-transformed individuals. It is, therefore, important that the transformants have an easily scored marker. The current favourites are the fluorescent proteins, such as GFP, DsRed and their mutant derivatives. These require transcriptional control elements, including a promoter, for their function. The best known of these are from the *Drosophila* Actin5C (Act5C) and ubi-p63E (Pub) genes. A silk moth homologue of Act5C, BmA3, has also been used, as well as a couple of tissue-specific promoters (3xP3, a synthetic eye-specific promoter, and Act88F, specific to the indirect flight muscles).

However, none of these promoters is entirely satisfactory. Act5C has been used to transform various mosquitoes, as well as *Drosophila*, but its expression pattern in mosquitoes is far from ubiquitous (Catteruccia *et al.*, 2000; Pinkerton *et al.*, 2000). Efforts to use it as part of a transformation marker in medfly (*Ceratitis capitata*) have failed, where equivalent experiments with Pub have achieved good success. Pub has similar limitations: the expression pattern seen in medfly transformants is highly variable, suggesting that the expression pattern is at least highly sensitive to position effect. In addition, none of these promoters can be regulated in the sense of being turned on and off as desired.

Fussenegger *et al.*, (1998a; 1998b) illustrate positive feedback driving multi-cistronic transcripts, using a selection marker, in one instance. Experiments were restricted to mammalian systems. pTRIDENT is described as a tricistronic artificial mammalian operon. Expression or

transient expression of cell cycle arresting genes is described for "metabolic engineering", *i.e.* regulating expression of desirable proteins, and it is mentioned that a transcriptional "squelching" effect by the VP16 transactivator domain may be lethal for the host cell, even at moderate expression levels (Berger *et al.*, 1990; Damke *et al.*, 1995; Gill and Ptashne, 1988; Gossen and Bujard, 1992; Salghetti *et al.*, 2001). The benefits of autoregulatory mono- or poly-cistronic systems are discussed, including one-step, auto-regulated and auto-selective multicistronic mammalian expression systems which included the tTA in a multicistronic, pTRIDENT-based or quattrocistronic configuration (pQuattro-tTA; Fussenegger *et al.*, (1998b); Figure 2). Since the tTA gene is encoded on the multicistronic expression unit itself, little or no tTA is expressed under repressive conditions. This positive feedback regulation system showed no signs of squelching. Experiments with a monocistronic positive feedback configuration in transgenic animals also showed no detrimental effects (Shockett *et al.*, 1995).

Very few promoters or other control elements have been characterised, and there remains a pressing need for such elements. It would be desirable to provide a universal promoter active in all or most cells of a wide range of insects, or to enable wider usage of an existing promoter. It is a further aim to regulate the activity of insect promoters, especially in a life stage- and/or sex-specific manner. It is also an aim to selectively reduce or eliminate the promoter activity in particular cells or tissues. The present invention provides such systems.

Surprisingly, it has now been found that it is possible to employ a positive feedback mechanism both to enhance the effect of an insect promoter, as well as to control its expression.

Thus, in a first aspect, the present invention provides an insect gene expression system, comprising at least one gene to be expressed and at least one promoter therefor, wherein a product of a gene to be expressed serves as a positive transcriptional control factor for the at least one promoter, and whereby the product, or the expression of the product, is controllable.

As used herein, the term "gene" refers to any DNA sequence that may transcribed or translated into a product, at least one such having activity or function *in vivo*. Such a gene will normally have at least a transcription promoter and a terminator operably associated therewith.

The product capable of positive transcriptional control may act in any suitable manner. In particular, the product may bind to an enhancer located in proximity to the promoter or promoters, thereby serving to enhance polymerase binding at the promoter, for example. Other mechanisms may be employed, such as repressor countering mechanisms, such as the blocking of an inhibitor of transcription or translation. Transcription inhibitors may be blocked, for example, by the use of hairpin RNA's or ribozymes to block translation of the mRNA encoding the inhibitor, for example, or the product may bind the inhibitor directly, thereby preventing inhibition of transcription or translation.

More preferably, the mechanism is a positive feedback mechanism, wherein the product, which may either be RNA or the translation product thereof, acts at a transcription enhancer site, normally by binding the site, thereby enhancing promoter activity. Enhancement of the promoter activity then serves to increase transcription of the gene for the product which, in turn, further serves to either lift inhibition or enhance promotion, thereby leading to a positive feedback loop.

Control of the product may be by any suitable means, and may be effective at any level. In particular, it is preferred that the control be effective either to block transcription of the control factor gene or to block translation of the RNA product thereof, or to prevent or inhibit action of the translation product of the gene.

For example, the gene product of tTA (tetracycline-repressible transcription activator) acts at the tetO operator sequence (Baron and Bujard, 2000; Gossen *et al.*, 1994; Gossen and Bujard, 1992). Upstream of a promoter, in either orientation, tetO is capable of enhancing levels of transcription from a promoter in close proximity thereto, when bound by the product of the tTA gene. If the tTA gene is part of the cassette comprising the tetO operator together with the promoter, then positive feedback occurs when the tTA gene product is expressed.

Control of this system is readily achieved by exposure to tetracycline, which binds to the gene product and prevents transactivation at tetO.

The tTA system also has the advantage of providing stage-specific toxicity in a number of species. In particular, "squelching" is observed in the development phases of many insects, the precise phase of susceptible insects being species-dependent. Some insects may reach

pupation before the larva dies, while others die early on. Susceptibility ranges from 100% fatality to a small reduction in survival rates. In general, though, adult insects appear to be immune to the squelching effect of tTA, so that it is possible to raise insects comprising a tTA positive feedback system in the presence of tetracycline, and then to release the adult insects into the wild. These insects are at little or no competitive disadvantage to the wild type, and will breed with the wild type insects, but larvae carrying the tTA positive feedback cassette will die before reaching maturity.

It is relatively straightforward to modify the tTA sequence to enhance compatibility with the desired insect species, and this has been demonstrated, in the accompanying Examples, with tTAV, which has an additional two amino acids to provide a protease site, but which is encoded by a sequence substantially changed from that of tTA in order to more closely follow *Drosophila* usage.

Accordingly, in a preferred aspect, the present invention provides a system as described, wherein at least one gene is tTA, or is a gene encoding a similar product to tTA effective to up-regulate the tetO promoter.

Thus, the present invention is useful in combination with a dominant lethal gene, allowing selective expression of the dominant lethal gene, or stage specific expression, as desired, of the lethal gene or the lethal phenotype. It will be appreciated that the dominant lethal gene does not need to be an integral part of the positive feedback mechanism, but may be part of a bicistronic cassette, for example. Use of the present invention in association with RIDL (Release of Insects carrying a Dominant Lethal) is particularly preferred.

Control of the feedback mechanism, in the case of tTA or an analogue thereof, is simply effected by the presence or absence of tetracycline, or by modulating tetracycline concentration, when the tTA gene product is used. In the case of another preferred positive feedback system, GAL4, this may be controlled by temperature, for example, thereby suppressing the effective gene, preferably a dominant lethal gene, until release of the insect.

Other mechanisms may also be employed, such as ribozymes or antisense or partially self-complementary RNA molecules, such as hairpin RNA, to inhibit or prevent expression of an activating peptide, or blocking agents that prevent binding of the activator to the enhancer site.

Such blocking agents may be expressed by the insect itself under selective conditions, or may be administered as part of the culture medium, for example.

Where the blocking, or controlling agents are produced by the insect, then it is preferred that their expression be selective, such as being sex specific. Administration of the blocking agent in the culture medium, for example, will enable suppression of the positive feedback cassette under all circumstances until release of the insect, after which stage- or sex- specific selection will occur, preferably in a succeeding generation, particularly preferably the following generation.

More preferably, the cassette comprising the positive feedback mechanism is associated with stage- or sex- specificity. For example, sex specific splicing is observed with the *transformer* and *doublesex* mechanisms seen in most insects, and can be employed to limit expression of the feedback system to a particular sex, either by employing sex specific splicing to delete all or part of the effector gene, or to incorporate a frameshift or stop codon, or to modulate RNA stability or mRNA translational efficiency, for example, or otherwise to affect expression so as to differentiate between the sexes. Targeting the females of pest species is particularly preferred.

Although it is possible to provide the effector gene in a separate location and even on a separate chromosome, it is generally preferable to link the effector gene with the feedback gene. This may be achieved either by placing the two genes in tandem, including the possibility of providing the two as a fusion product, or for example by providing each gene with its own promoter in opposite orientations but in juxtaposition to the enhancer site.

An effector gene is the gene whose expression it is desired to enhance. Where a positive feedback product is also effective as a stage-specific lethal, such as tTA in many species, then the effector and the feedback gene may be one and the same, and this is a preferred embodiment.

The effector gene will often be a lethal gene, and it is envisaged that the system of the present invention will most frequently be employed in the control of insect pest populations, particularly in combination with the RIDL technique or related method, as described hereinunder.

It is preferred to include a marker with the systems of the invention, such as DsRed, green fluorescent protein, and variants thereon, as transformation success rates in insects are extremely low, so that it is useful to be able to select in some way.

The promoter may be a large or complex promoter, but these often suffer the disadvantage of being poorly or patchily utilised when introduced into non-host insects. Accordingly, it is preferred to employ minimal promoters, such as the Hsp70 promoter which, while having a naturally somewhat low level of activity, can be substantially enhanced by a positive feedback scenario, such as by the use of tTA and tetO.

A promoter is a DNA sequence, generally directly upstream to the coding sequence, required for basal and/or regulated transcription of a gene. In particular, a promoter has sufficient information to allow initiation of transcription, generally having a transcription initiation start site and a binding site for the polymerase complex. A minimal promoter will generally have sufficient additional sequence to permit these two to be effective. Other sequence information, such as that which determines tissue specificity, for example, is usually lacking, and preferred minimal promoters are, normally as a direct result of this deficiency, substantially inactive in the absence of an active enhancer. Thus, a cistron, or system, the two terms preferably being generally interchangeable herein, of the invention will generally be inactive when the or each promoter is a minimal promoter, until a suitable enhancer or other regulatory element is de-repressed or activated, typically the gene product.

Thus, it will be appreciated that minimal promoters may be obtained directly from known sources of promoters, or derived from larger naturally occurring, or otherwise known, promoters. Suitable minimal promoters and how to obtain them will be readily apparent to those skilled in the art. For example, suitable minimal promoters include a minimal promoter derived from hsp70, a P minimal promoter (exemplified hereinunder as WTP-tTA), a CMV minimal promoter (exemplified hereinunder as JY2004-tTA), an Act5C-based minimal promoter, a BmA3

promoter fragment, and an Adh core promoter (Bieschke, E., Wheeler, J., and Tower, J. (1998). Doxycycline-induced transgene expression during Drosophila development and aging. Mol Gen Genet 258, 571-579). Act5C responds to tTA in transgenic *Aedes*, for example, and the invention.

Not all minimal promoters will necessarily work in all species of insect, but it is readily apparent to those skilled in the art as to how to ensure that the promoter is active. For example, a plasmid, or other vector, comprising a cistron of the invention with the minimal promoter to be tested further comprises a marker, such a gene encoding a fluorescent protein, under the control of a promoter known to work in that species, the method further comprising assaying putative transgenic individuals for expression of the marker, and wherein individuals expressing the marker are then assayed for expression of the gene under the control of the minimal promoter, such as by assaying transcribed RNA. Presence of the RNA above background levels under induced or de-repressed conditions is indicative that the minimal promoter is active in the species under investigation; absence or presence at low levels only of such RNA in non-induced or repressed conditions is indicative that the minimal promoter has low intrinsic basal activity.

We have used the following marker promoters, by way of example, only, but many more are useful and apparent to those skilled in the art:

mini-white (white promoter): WTP2-tTA, JY2004-tTA
Act5C promoter: LA513 and LA517
ubi-p63E promoter: LA656 and LA1038
BmA3 promoter: LA710
hr enhancer and ie1 promoter: LA928, LA1124 and LA1188
and all of these are useful as, or in the preparation of, minimal promoters.

It will be appreciated that a cistron or system of the invention may comprise two or more cistrons. A system may further comprise non-linked elements, such as where a second gene to be expressed is remote from the positive feedback cistron.

Thus, in a preferred aspect, the present invention provides positive feedback constructs of the general form shown in accompanying Figure 1. In this scenario, the tetracycline-repressible transcription activator (tTA) protein, when expressed, binds to the tetO operator sequence and

drives expression from a nearby minimal promoter. In the configuration shown, this then drives expression of tTA, which then binds to tetO, and so on, creating a positive feedback system. This system is inhibited by tetracycline, which binds to tTA and prevents it binding tetO.

Expression is controllable, and this may be achieved by operably linking the promoter to a controllable transcription factor. As illustrated above, this may be tTA (tetracycline-repressible or tetracycline-inducible), or any other factor controllable system, such as GAL4 (which is somewhat cold-sensitive, and can be further controlled by use of GAL80 or mutants thereof), or the streptogrammin regulated expression system, for example. It will be appreciated that other binding sites for the appropriate transcription factor will depend on the transcription factor concerned, such as UAS_{GAL4} (upstream activation sequence) for GAL4, for example.

Preferred systems of the present invention have high levels of induced expression, preferably available at several induced levels, with a low basal level of expression of the regulated gene but also of any other component, and preferably across a range of species. Basal levels are preferably low or substantially non-existent where expression is strongly deleterious, but acceptable levels will depend on the effect of the product. Maximum levels will not generally be an issue, as the positive feedback condition will often provide fatal levels of expression and, even where the expression product is not fatal, or associated with fatal consequences, it is likely to be expressed in far higher concentrations than most gene products.

Where a basal level of expression is desired, then a promoter sequence that does not need the presence of the enhancer may be employed, although there will then, generally, be feedback. Unless there is a cut-off level of feedback, below which the feedback product will not work, then it will be appreciated that it is preferred to keep to a minimum feedback gene expression

Different constructs of the invention (described in the accompanying Examples) have varying activity, according to the components of the constructs. For example, in *Drosophila*:
WTP-tTA gives a low level of induced (non-repressed) expression
JY2004-tTA gives strong expression when not repressed, approximately equivalent to Act5C-tTA
LA513 is lethal when not repressed.

The first two appear to give constitutive expression, as judged by use of a reporter gene (tRE-EGFP), this is difficult to assess for the lethal LA513, although at 10μg/ml tet, just sufficient for good survival, LA513 in *Drosophila* drives expression of a tetO$_7$-EGFP reporter gene in both the male and female germline in adults, as well as in somatic cells. This distinguishes it from Act5C, commonly used as a "ubiquitous, constitutive" promoter, which does not, in fact, express well in these cells.

The properties of these constructs are shown in Table 1, below.

Table 1

	Max expression	Minimal promoter	Intron	Optimised coding region?	3'UTR and polyA
WTP-tTA	Low	P	PP1α96A	No	*fs(1)K10*
JY2004-tTA	High	CMV	Rabbit β-globin	No	Rabbit β-globin
LA513	V. high (lethal)	Hsp70	Adh	Yes	*fs(1)K10*

Accordingly, it will be appreciated that the induced or non-repressed expression level can be modified in a useful and predictable way by adjusting the sequence of the positive feedback system. Toxicity and/or activity of the tTA protein can be modified independently of the transcriptional and translational control signals by several approaches, *e.g.* use of a nuclear localisation signal, modification of the activation domain, *etc.* (see Fussenegger, 2001 for more examples).

The lethality of LA513 is useful, for the reasons given above, and more particularly because:

a) It provides a compact, highly effective repressible lethal gene system;

b) As it uses only simple control elements from *Drosophila* (hsp70 minimal promoter, a small intron and a terminator from *fs(1)K10*), it, or its expression cassette, functions across a wide phylogenetic range;

c) It has very little, if any, deleterious effect on adults, even in the absence of tetracycline. This is a highly desirable and surprising property for field use, for example in a RIDL-based control programme, as the released adults must be competitive and long-lived for full efficacy of

the programme. It will be appreciated that the effect of the system of the invention could be further modified by the incorporation of an adult-effective lethal, for example in the "positive feedback – bi-directional expression" configuration described herein; and

d) By its nature, "cross-talk" between various elements is minimised. This is because: (i) the core of the construct is only a single composite element, rather than the normal two in bipartite expression systems; (ii) the principal enhancer of the autoregulatory component, the tTA binding sites, is substantially active only in the absence of tetracycline and (iii) modest expression of tTA under the influence of a nearby enhancer, whether in another part of the construct or in nearby chromatin, is unlikely to be significantly deleterious.

JY2004-tTA is also useful, in the present invention.

Without being bound by theory, the mechanism by which LA513 kills embryos and early larvae, but not adults, appears to be an inherent property of its toxicity. tTA toxicity is believed to derive from "transcriptional squelching", in which high level expression of the transcriptional activator domain (in the case of tTA this is VP16 or a fragment thereof) binds elements of the transcriptional machinery and titrates them, leading to a general effect on transcription, although it may also act to saturate the ubiquitin degradation pathway. Transcriptional squelching is the effect which is thought to lead to deleterious effects in mammalian cell lines expressing tTA at high levels; in the optimised expression context of LA513 positive feedback drives tTA expression to lethal levels. However, developing stages may be more sensitive to disruption of transcription than adults: they have to express genes in a highly coordinated fashion to allow proper development, while adults may be more tolerant of disruption.

The development of LA513 heterozygotes on media with an intermediate level of tet (3 or 10 μg/ml), just sufficient for survival, showed a significant delay, relative to their wild type siblings. Parallel experiments using higher concentrations of tetracycline, *e.g.* 100 μg/ml, did not show any developmental delay, thereby suggesting that sub-lethal expression of tTA can adversely affect the normal development of the insects.

It is preferred that a positive feedback system show a higher on:off ratio and switch from on to off over a narrower concentration range than a conventional system, thereby allowing the use of a wider range of effector molecules. Lower-toxicity (lower specific activity) effector

molecules can be used, as they can be expressed at a high level under active conditions without leading to problems of toxicity at basal levels. Conversely, more toxic (higher specific activity) ones can be used as the necessary low basal level does not preclude high levels of expression when de-repressed or induced. Since basal level of expression is only partly determined by the level of tTA, this advantage is particularly clear in the case of lower-toxicity molecules. tTA is a preferred example of a low specific activity effector molecule that can be used as a lethal in the positive feedback context of LA513, for example. The advantage of switching from on to off over a narrow concentration range is that a modest concentration of repressor can be used without risk of residual (not fully repressed) expression leading to adverse effects and potentially selecting for resistance. Conversely, for an inducible system, modest concentrations of the activator can give full expression.

Activated or de-repressed drivers are useful for expressing effector molecules. Examples of effector molecules include functional RNA's, such as hairpin RNA's, ribozymes *etc.*, and one or more encoded proteins. It will be appreciated that, for different applications, different levels of expression are appropriate. Since the sequence-specific transcription factors used to drive the positive feedback system can also be used to express other genes in a bipartite expression system, this may be achieved by making two separate constructs, one with the driver (normally a promoter-transcription factor construct, here the positive feedback construct), the other with the gene or molecule of interest under the control of a composite promoter (binding site + minimal promoter) responsive to the transcription factor (Bello *et al.*, 1998; Brand *et al.*, 1994). This is also appropriate for these positive feedback drivers. Alternatively, the two elements may be combined on the same construct. This embodiment has significant advantages for most field applications, as it very substantially reduces the risk that the two functional elements can be separated by recombination. Further, the complete expression system can be introduced with only a single transformation event, as well as meaning that insects homozygous for the system are homozygous at only one locus rather than two, which makes them easier to construct by breeding, and tends to reduce the fitness cost due to insertional mutagenesis.

It is also possible to condense such an expression system into a more compact form, such as is illustrated in accompanying Figure 2.

This exploits the bi-directional nature of enhancers, in this case the tetO binding site in the presence of tTA. This arrangement further allows, or facilitates, the use of insulator elements to reduce the effect of enhancers or suppressors in the adjacent chromatin: in this arrangement the entire expression cassette can be flanked by insulators. This arrangement also removes the need to duplicate the transcription factor binding sites within the construct. Such duplication is preferably avoided, as it can lead to instability through homologous recombination. For similar reasons, it is generally preferred that non-identical insulators, such as scs and scs' are used, rather than using the same one twice.

It is further possible to condense the system to provide a single transcript, either bicistronic or expressing a single polypeptide, which may potentially be further processed into more than one protein, for example by use of the ubiquitin fusion technique (Varshavsky, 2000). Each of these approaches (bi-directional expression, bicistronic expression, fusion protein with transactivator) tends to reduce the size of the construct, which in turn will tend to increase the transformation frequency and reduce the mutagenic target. Such condensation can be achieved in several ways, as shown, diagrammatically, in accompanying Figure 3. Appropriate extensions to and variations of the arrangements shown diagrammatically will be apparent to those skilled in the art.

As an example of the utility of such a system, a general transformation marker might be constructed by using a transactivator system known to function over a wide phylogenetic range, for example those based on tetR, GAL4, lexA or AcNPV ie-1. Such a transactivator, functionally linked to a coding region for a fluorescent protein by any of the above methods (bi-directional expression, bicistronic expression, fusion protein with transactivator), would provide a genetic marker expressed in a wide range of tissues and developmental stages across a broad phylogenetic range. Such a marker would be useful not only for detecting transgenics in transformation and other lab experiments, but also for distinguishing, for example, transgenic flies from wild type flies in the field, or those caught in the field.

Another example is expression of a transposase. Integrated into the chromosomes, this would be a "jump-starter" construct, for example *piggyBac* transposase integrated into an insect chromosome using *mariner/mos1*. Such constructs are useful to remobilise *piggyBac* elements. A widely-applicable jump-starter should be expressed at a significant level across a wide

phylogenetic range. The expression system of this invention provides this. Furthermore, such a construct (*piggyBac* transposase under the control of a positive feedback system of one of the above structures) would also be useful in insect transformation *via* transient expression (co-expression of a "helper" plasmid, the most widely-used method for insect transformation), and again would be useful and functional across a wide phylogenetic range.

It is advantageous to regulate the action of an expression system at stage-, sex- or other levels, in addition to being able to regulate the expression level by changing environmental conditions. Suitable examples are as follows:

1. Expression of a repressor protein.

Repressor proteins are known or can be constructed for the main expression systems, *e.g.* GAL80 or its mutant derivatives for the GAL4 system, tetR fused to inhibitory proteins for the tet system, *etc.* Another alternative is gene silencing of the transcription factor using a hairpin RNA directed against part of the expression cassette. Basal expression from the positive feedback system is rather low, therefore it can readily be suppressed by expression of such an inhibitor.

Expression of a suitable inhibitor under suitable control will tend to inhibit expression from the positive feedback expression cassette where the inhibitor is expressed. Female-specific expression, for example, can therefore be achieved by expressing an inhibitor in males.

2. Integrating specificity into the positive feedback system.

Specificity can be integrated into the positive feedback system by using components that are themselves specific. For example, the hsp70 minimal promoter + SV40 intron and polyA signal combination of pUAST is known not to be expressed in the female germline of *Drosophila*, while the P minimal promoter + P intron + *fs(1)K10* polyA signal of pUASp is so expressed (Rorth, 1998). Positive feedback expression systems can, therefore, be constructed which specifically do or do not express in this tissue, depending on the use of appropriate regulatory elements.

In another embodiment, sex-specificity can be integrated into the system by use of sex-specific splicing. The sex-specific splicing of *doublesex* and its homologues is a conserved regulatory mechanism and, therefore, available for use in this way across a wide phylogenetic range. Sex-specific splicing of *transformer* and its homologues is another alternative. The use of sex-

specific splicing to integrate specificity into a positive feedback expression system can be achieved in several ways, as shown, diagrammatically, in accompanying Figure 4. Appropriate extensions to and variations of the arrangements shown diagrammatically will be apparent to those skilled in the art.

In another configuration, a specific splice site can be inserted into the transactivator coding region so that two (or more) alternative proteins are produced in different conditions, *e.g.* in different cell types or in different sexes. This can be arranged so that a transcriptional activator is produced in one cell type but a transcriptional repressor is produced in another cell type. This arrangement has the advantage that it is relatively robust to inefficient (imperfect) splicing – production of a relatively low proportion of transcriptional activator in the inappropriate cell type, *e.g.* in male cells, will be less likely to produce the positive feedback amplification as these cells are also producing a larger amount of repressor. Discrimination in output (ratio of levels of transcriptional activator in the two cell types, or ratio of expression of a reporter or other RNA or protein functionally linked to the expression of the transcriptional activator) between the two cell types is thereby enhanced.

It will be readily apparent to those skilled in the art that any of these specific transactivator arrangements can readily be combined with any of the arrangements disclosed herein for expression of an additional protein or RNA, *e.g.* bi-directional expression, bi- or multi-cistronic expression, expression of a fusion protein, or combined with one or more separate expression cassettes dependent on, or partly dependent on, expression of the transactivator, either combined on the same construct or elsewhere in the genome or cell.

3. Using a specific effector molecule

Specificity in phenotypic consequence can also be introduced by use of a specific effector molecule. Where a molecule, *e.g.* RNA or protein, expressed under the control of any of the expression systems described herein, has a specific effect only in particular cells, tissues, or sex, *etc*, then phenotypic specificity can be obtained with broader or less specific expression of the transactivator. For example, in the context of a RIDL-type mass-release insect population control programme, using the system to express a molecule only toxic, or preferentially toxic, to pre-adult stages, results in adults which are fully, or reasonably competitive, relative to wild type. This is desirable as the effectiveness of the programme depends on the competitiveness

and longevity of the adult forms, when released into the wild. Since their internal repressor (*e.g.* tetracycline) concentration is likely to decline in the wild, it would be advantageous to ensure that induction (de-repression) of the expression system, as and when it occurs in adults, has a minimal negative effect on them.

As another example, sex separation, or sex-specific effects, can be achieved by expression in both males and females of a molecule with differential effects in males and females. For example, expression of the Transformer protein in male *Drosophila* will tend to transform them into females, but have no effect on females. Similarly, expression of Male specific lethal-2 (Msl-2) protein in *Drosophila* will tend to kill females, but not males (Gebauer *et al.*, 1998; Kelley *et al.*, 1995; Matsuo *et al.*, 1997; Thomas *et al.*, 2000). Conversely, expression of a partially self-complementary RNA molecule with substantial homology in its self-complementary or double-strand-forming region to ("hairpin RNA against") *transformer* will tend to transform genetic females into phenotypic males, while not affecting genetic males, and expression of hairpin RNA against *msl-2* will tend to be lethal to males but not to females. Expression of hairpin RNA against the male- or female-specific exons of *doublesex* will tend to affect those sexes only, and simultaneous expression of RNA encoding the other form of *doublesex* (*i.e.* Dsx^M in females or Dsx^F in males) will tend to modify or enhance this effect. This simultaneous expression of a protein and a hairpin RNA molecule can readily be accomplished by combining the bicistronic or fusion protein approach described above with expression of a hairpin RNA using the bi-directional expression system also described above. Sex-, stage- or other specificity can be further added to such a system by incorporation of appropriate specific splicing or other transcriptional, translational or other post-translational control signals to either part of the system as will be apparent to the person skilled in the art.

Multi-functional hairpin RNA molecules may be constructed and are envisaged. For example, RNAi against *transformer* in the Mediterranean fruit fly *Ceratitis capitata* Wiedmann (medfly) will tend to transform genetic females into fertile males. For an area-wide population control program based on mass-release of such insects, it is preferable to sterilise the released flies. This can be accomplished by using a composite RNA molecule that simultaneously disrupts expression of both *transformer* and a gene required for spermatogenesis or embryonic or larval viability. Many such genes are known in *Drosophila* with homologues in mosquitoes or other animals. With medfly, a suitable homologue can readily be isolated, using techniques

known to those skilled in the art. We prefer the use of a gene which allows the production of seminal fluid, and preferably also of sperm, to reduce the tendency of the female to re-mate after insemination by the affected male. We particularly prefer to direct this second part of the hairpin RNAi molecule against a paternal effect lethal, so that no viable progeny can be produced, or against a zygotically expressed gene required for embryonic or larval viability or development, so that progeny inheriting the construct will be affected. Other configurations are envisioned and will be readily apparent to those skilled in the art: for example expression of a female-specific lethal protein by bicistronic expression and a hairpin RNA leading to paternal-effect lethality by bi-directional expression. In common with the composite hairpin RNA against a suitable sex-determination gene and a paternal effect lethal, this allows the generation of a single-sex (male-only) population of insects, all of whose progeny die through the action of the paternal-effect lethal, irrespective of whether their progeny or mates feed on tetracycline. Thus, the present invention provides a controlled promoter, as defined, wherein the promoter is operably linked with DNA encoding an RNAi causing lethality or sterility. In this case, lethality may correspond to low fitness, such as flightless, rather than outright lethality, provided that the likelihood of breeding on is substantially reduced.

4. Using site-specific recombinase(s)

Specificity can also be introduced into the positive feedback system by inserting a "stuffer" fragment which inactivates it. If this "stuffer" fragment is flanked by target sites for a suitable site-specific recombinase, then it will tend to be excised in the presence of active recombinase. Any system for selective expression of active recombinase, for example, expression of the recombinase under the control of a female-specific promoter, will therefore tend to lead to selective expression of the positive feedback system, in this case in females only. If the recombinase is expressed in somatic cells only, for example by using the method described above, then the version transmitted to the next generation includes the stuffer fragment, which can again be daughters but not sons. Conversely, if the recombinase is expressed in the genome only, provision of active recombinase will lead to offspring in which the expression system is active, from parents in which it is inactive. This can be used, for example, to generate gametes containing an active dominant lethal or sterile gene system (*e.g.* female-specific or non-sex-specific) for use in an insect population control strategy.

In a preferred embodiment, the stuffer fragment encodes the recombinase. This embodiment is particularly compact. In another preferred embodiment, the stuffer fragment encodes a transcriptional repressor which tends to inactivate the positive feedback expression system – this embodiment tends to reduce the basal expression of the system in the presence of the stuffer fragment.

Conversely, the system can be specifically inactivated in certain cells, or clones of cells, by introducing target sites for a suitable site-specific recombinase at suitable positions, and then expressing or introducing the appropriate active recombinase in appropriate cells, such that one or more key functional elements of the expression system are removed or disrupted by recombination between the target sites for the recombinase.

Suitable recombinase systems include cre/lox and Flp/FRT.

The present invention is illustrated by the following, non-limiting, Examples. In the following Examples, the Figures are as follows:
Figure 1 shows a tetracycline-repressible transcription activator scenario;
Figure 2 shows a system of the invention using a bi-directional enhancer;
Figure 3 shows a sex-specific system;
Figure 4 shows another sex-specific system;
Figure 5 is a diagram of the tetO$_7$-tTA region of pJY2004;
Figure 6 is a schematic diagram of pLA513;
Figure 7 is a schematic diagram of the LA513 transposon;
Figure 8 is a schematic diagram of pLA517;
Figure 9 illustrates the bidirectional action of tetO$_7$ in 513A and 513B mosquitoes;
Figure 10 is a schematic diagram of pLA656;
Figure 11 is a schematic diagram of pLA928;
Figure 12 is a schematic diagram of pLA1124;
Figure 13 is a schematic diagram of pLA670;
Figure 14 is a schematic diagram of pLA1038;
Figure 15 is a schematic diagram of pLA710;
Figure 16 illustrates the sex-specific splicing of *Cctra* in medfly;
Figure 17 is a schematic diagram of pLA1188; and

Figure 18 illustrates sex-specific splicing in medfly.

EXAMPLES

A series of constructs was made with tTA in a positive feedback configuration, *i.e.* with tTA expression regulated by tTA binding to tetO. Transgenic insects carrying these constructs were obtained and their properties analysed.

tTAV

In some cases, the intention was to obtain very high levels of expression of tTA in the absence of tetracycline. In various exemplified constructs described hereinbelow, tTA expression was so high as to be lethal. As part of the process of obtaining strong expression of tTA, part of the tTA open reading frame was redesigned to express a similar protein, but with codon usage closer to the norm for *Drosophila melanogaster*, and lacking some potential cryptic splice sites present in the original nucleotide sequence. This variant tTA sequence was named tTAV (SEQ ID NO. 31, protein sequence SEQ ID NO. 32).

EXAMPLE 1

WTP-tTA and JY2004-tTA in *Drosophila melanogaster*

The tTA coding region (SEQ ID NO. 29, tTA protein sequence SEQ ID NO. 30) from pUHD15-1 (SEQ ID NO. 33, Gossen *et al.*, 1994; Gossen and Bujard, 1992) was placed under tetO control, in a positive feedback configuration, by inserting it into pWTP2 (Bello *et al.*, 1998) or pJY2004, a version of pJY2000 that lacks insulators (Stebbins and Yin, 2001). These constructs were named pWTP-tTA and pJY2004-tTA, respectively. A diagram of $tetO_7$-tTA region of pJY2004 is provided as accompanying Figure 5, and is SEQ ID NO. 14.

In pWTP-tTA, the $tetO_7$ binding sites are followed by a minimal promoter from the *P* element, a leader sequence from *Drosophila hsp70,* a short intron from the *Drosophila* PP1α96A gene, the tTA coding region and a transcription terminator from *Drosophila hsp70*. In pJY2004-tTA, the minimal promoter and leader sequences are from CMV, followed by the tTA coding region and a transcription terminator from rabbit β-globin, as shown in Figure 5.

Transgenic *Drosophila melanogaster* carrying either of these constructs were fully viable, even without dietary tetracycline. Insects doubly heterozygous for WTP-EGFP and either of these constructs were examined for green fluorescence characteristic of EGFP expression. Insects with WTP-tTA and WTP-EGFP showed very weak fluorescence only slightly above background autofluorescence. In contrast, insects with JY2004-tTA and WTP-EGFP showed strong fluorescence, similar to that seen in insects carrying EGFP under the control of the Actin5C promoter, which is widely used as a strong, constitutive promoter in *Drosophila* (*e.g.* Reichhart and Ferrandon, 1998). Expression of EGFP was repressed to undetectable levels when the insects were raised on diet supplemented with tetracycline to 100 μg/ml. Control insects heterozygous for either WTP-EGFP, JY2004-tTA or WTP-tTA showed no fluorescence above background whether or not they were raised on a diet containing tetracycline.

We placed tTA under the control of the Actin5C promoter, in plasmid pP [Casper-Act5C-tTA]. Transgenic flies carrying this construct and WTP-EGFP, raised on a diet lacking tetracycline, showed green fluorescence at a comparable intensity to that observed in equivalent flies with JY2004-tTA and WTP-EGFP.

These results show that positive feedback constructs can be used to give strong (JY2004-tTA) or weak (WTP-tTA), tetracycline-repressible expression from a suitable construct (here WTP-EGFP).

EGFP is widely used as a neutral reporter. We further tested JY2004-tTA flies by crossing them to flies with constructs capable of expressing proteins known or predicted to be deleterious. We inserted the central domain of Nipp1Dm (Bennett *et al.*, 2003; Parker *et al.*, 2002) ("nipper"), into pJY2004, to make pJY2004-nipper, and transformed *Drosophila* with this construct. We also used flies carrying tetO-hid (Heinrich and Scott, 2000). In each case,

crossing to JY2004-tTA flies gave tetracycline-repressible lethality. Data from two example crosses are presented in Table 2, below.

Table 2

Use of positive feedback constructs to drive expression of lethal genes in *Drosophila*.

Male JY2004-tTA/CyO x Female tetO-hid/tetO-hid				[tetracycline] (μg/ml)
JY2004-tTA	CyO			
0	15			0
9	10			100
Male JY2004-tTA/CyO x Female JY2004-nipper/JY2004-nipper				
JY2004-tTA	CyO			
0	20			0
16	13			100

EXAMPLE 2

LA513 in *Drosophila melanogaster*

We made construct pLA513 (SEQ ID NO. 16, schematic diagram shown in Figure 6), containing a non-autonomous *piggyBac* transposon. We generated transgenic *Drosophila melanogaster* carrying this construct by co-injection with a helper plasmid into a white-eyed strain (Handler, 2002; Handler and James, 2000). Potential transgenics were screened for fluorescence characteristic of DsRed2. 5 transgenic lines were recovered, and were designated O513, M8, M13, F23 and F24. A schematic diagram of the LA513 transposon is shown in accompanying Figure 7.

Drosophila melanogaster stocks were maintained at 25°C on yeast/sugar/maize/ tetracycline medium (tetracycline (Sigma) at 100μg/ml final concentration), unless stated otherwise. All experiments were performed at 25°C.

Survival of LA513/+ transgenics with and without tetracycline

Heterozygous transgenics were crossed in at least triplicate to wild type on media with or without Tc (tetracycline). In the absence of any lethality, it would be expected that approximately half the progeny of such a cross would be transgenic. Progeny were scored as young adults for DsRed marker fluorescence [Matz *et al.*, 1999] using an Olympus SZX12 microscope with fluorescence capability, and the ratio of fluorescent (transgenic) to total flies was calculated. The results are shown in Table 3, below. In these experiments, all 5 transgenic lines showed 100% lethality, in the absence of tetracycline, and good survival (*i.e.* fluorescent:non-fluorescent ratio ~1:1), in the presence of 100μg/ml tetracycline. Inspection of the vials showed few or no large fluorescent larvae in the absence of Tc, although many very small fluorescent larvae were present, at a time when non-fluorescent (wild type for LA513) larvae were visible at all sizes. This suggests that, in the absence of tetracycline, LA513 causes lethality at an early (embryonic and/or early larval) developmental stage.

Table 3

LA513 insertions are tetracycline-repressible dominant lethals

	0 μg/ml tetracycline		**100 μg/ml tetracycline**		
LA513 line	# Flies	# Fluorescent	# Flies	# Fluorescent	Ratio
O513	490	0	1963	937	0.48
M8	74	0	66	25	0.38
M13	657	0	1838	892	0.49
F23	473	0	1914	845	0.44
F24	61	0	114	60	0.53
Total	*1755*	*0*	*5895*	*2759*	*0.47*

Dominant lethality could have several causes. Without being restricted by theory, it seems likely that, in the absence of tetracycline, tTAV accumulates to a relatively high concentration and that this is lethal, possibly due to transcriptional squelching, or interference with protein degradation. An alternative is that, in the absence of tetracycline, tTAV binds to tetO and acts as a long-range enhancer, perturbing the expression of genes near to the LA513 insertion. This appears unlikely, as all 5 transgenic lines gave similar results. Each of these lines was derived from a different G0 injection survivor, and these lines are, therefore, likely to carry LA513 integrated at different genomic sites. We verified this by inverse PCR. Table 4, below, shows the integration sites for 3 of the lines; in each case the LA513 insertion was at a TTAA sequence, as expected from the known insertion site preference of the *piggyBac* transposon. As expected, the 3 insertions were indeed at 3 different sites in the *Drosophila* genome.

Table 4

Insertion sites of LA513 in *Drosophila* genome

Line	**Sequence Amplified or at Site of Integration**	*Predicted chromosome arm*	***Predicted* Drosophila *cytology***	**Nearest predicted gene**
O513	Cacagcgcatgatgagcaca**TTAA**caaaatgtagtaaaatagga (SEQ ID NO. 1)	2L	25F4-25F5	CG9171
M8	Gtttcgataaatattgctat**TTAA**aatgcttattttcaatgcta (SEQ ID NO. 2)	2L	36F6–36F6	CG15160
F24	Tttgttttctaacgttaaag**TTAA**agagagtccagccacatttt (SEQ ID NO. 3)	2L	21C4-21C5	CG13691

Flanking sequence is shown with the TTAA insertion site capitalised. Predicted chromosome locations, and the nearest predicted gene, are also shown; these are based on the published *Drosophila* genome sequence.

EXAMPLE 3

Reducing the toxicity of tTAV

The toxic effect of high level expression of tTAV is thought to be due to transcriptional squelching and/or interference with ubiquitin-dependent proteolysis, *via* the VP16-derived section (Gossen and Bujard, 1992; Salghetti *et al.*, 2001). We, therefore, modified tTAV by removing the VP16 section and replacing it with a synthetic sequence which encodes 3 copies of a peptide (PADALDDFDLDML) derived from VP16 (Baron and Bujard, 2000; Baron *et al.*, 1997). This derivative was named tTAF; the resulting plasmid was named pLA517, and is SEQ ID NO. 17, and is shown, diagrammatically, in accompanying Figure 8.

Drosophila melanogaster were transformed with this construct, and one transgenic line was obtained. LA513 heterozygous males were crossed to wild type (for LA513) females and the progeny scored for fluorescence (as adults). If all progeny are equally likely to survive, the expected proportion of the total progeny that are fluorescent is 50%. In the absence of tetracycline, this proportion was 32%, only a modest reduction compared with 48% when parents and progeny were raised on diet supplemented with tetracycline to 100 μg/ml. The results are shown in Table 5, below. We tested whether supplying tetracycline in the diet of the parents but not of the progeny could reduce this lethality. In this case, we observed an intermediate proportion of 0.37, indicating that maternally contributed tetracycline has a modest beneficial effect.

Table 5

Effect of tetracycline on the survival of LA517/+ *Drosophila* and their +/+ siblings

LA517			
Parent [Tc] μg/ml	**Progeny [Tc]** μg/ml	**Non-Fluorescent**	**Fluorescent**
0	0	165	78
100	100	524	482
100	0	502	297

Since LA517, alone, had little impact on viability, unlike the closely related construct LA513, we tested whether it was capable of driving expression of a heterologous gene under tetO control. For this we used tetO-hid (Heinrich and Scott, 2000). Flies homozygous for tetO-hid were crossed with flies heterozygous for LA517. In the absence of tetracycline, only 3.4% of the adult progeny carried LA517. In the presence of 100 μg/ml tetracycline, this proportion was 42%. LA517 is, therefore, capable of driving effective expression of a heterologous gene.

Table 6

Effect of tetracycline on the survival of LA517/+, +/tetO-hid *Drosophila* and their +/+, +/tetO-hid siblings

TetO-Hid x LA517/+		
[Tc]	**Non-Fluorescent**	**Fluorescent**
0	636	23
100	174	127

EXAMPLE 4

Use of analogues of tetracycline

Line F23 was used to determine whether chemical analogues of tetracycline could be used in place of tetracycline to suppress the lethality of LA513. For this purpose we tested 3 analogues at a range of concentrations from 0 to 100 μg/ml (suppliers: tetracycline and doxycycline, Sigma; 4-epi-oxytetracycline, Acros Organics; chlortetracycline Fuzhou Antibiotic Group Corp.). We calculated the concentrations required for half-maximal survival. These are shown in Table 7, below.

Table 7

Efficacy of Tc analogues

Line	**Tc/Analogue**	**Concentration for half-maximal survival, μg/ml**
F23	Tetracycline	5.0
F23	Doxycycline	3.9
F23	7-chlortetracycline	1.7
F23	4-epi-oxytetracycline	42.0

EXAMPLE 5

Longevity of LA513/+ adults in the absence of tetracycline

LA513 clearly confers dominant lethality, active at an embryonic and/or early larval stage. Larvae were raised on a diet supplemented with 100μg/ml tetracycline. After eclosion, adults were transferred to a diet lacking tetracycline. The lifespan of these adults was measured, and also of comparable w^{1118} non-transgenic adults. As shown in Table 8, below, the transgenic lines showed good adult survival relative to the non-transgenic control. This suggests that stage-specificity can be obtained in this way – here LA513 is a larval/embryonic lethal, but not an adult lethal.

Table 8

Mean adult lifespan of LA513/+ transgenic *Drosophila*.

Line	**Mean post-eclosion survival time,** days	**Standard deviation**	**Number of Flies**
O513	40.3	12.3	66
M8	26.1	2.5	9
M13	29.5	9.9	47
F23	29.6	11.3	83
F24	19.9	10.0	9
w^{1118}	22.2	8.6	88

It is possible to explain these longevity data by postulating that larvae accumulate tetracycline by feeding, and retain this tetracycline into adulthood, so that they survive even in the absence of dietary tetracycline as adults. To examine this, flies heterozygous for LA513/+ (M13 line) were raised as larvae on various concentrations of tetracycline. After eclosion, adults were transferred to diet lacking tetracycline and the lifespan of these adults was measured, as above. As shown in Table 9, below, the concentration of dietary tetracycline as larvae had no obvious effect on subsequent adult longevity in the absence of tetracycline, implying that adult survival is not primarily due to retention of tetracycline from larval feeding. At a concentration of 1 μg / ml, no transgenics survived to adulthood, and at 3 μg/ml only about half of the expected number survived to adulthood, so that this concentration is close to the minimum for larval survival.

Table 9

Effect of larval tetracycline on adult longevity

Larval tetracycline μg / ml	Mean post-eclosion survival time, days	Standard deviation	Number of Flies
1	-	-	-
3	33.5	13.2	9
10	28.4	9.6	17
30	26.3	11.3	23
100	29.5	9.9	47

Another possible explanation for the survival of LA513/+ adults is that tTAV is inactive in adults, so that the positive feedback cycle does not work, and tTAV does not accumulate. We examined this by measuring the amount of tTAV mRNA by quantitative PCR following a reverse transcriptase reaction (quantitative rt-PCR, or qPCR). We used Taqman chemistry and reagents (ABI), and an ABI Prism 7000 qPCR instrument. Each sample was assayed in triplicate; data are the mean of these three assays. The 18S primers anneal to *Drosophila melanogaster*, *Ceratitis capitata* and *Aedes aegypti* 18S RNA, so these primers were used for all three species.

Primers used:

18S RNA		SEQ ID NO.
Forward Primer:	ACGCGAGAGGTGAAATTCTTG	4
Reverse Primer:	GAAAACATCTTTGGCAAATGCTT	5
TaqMan MGB Probe:	6-Fam-CCGTCGTAAGACTAAC-MGB	6
tTAV		
Forward Primer:	CATGCCGACGCGCTAGA	7
Reverse Primer:	GTAAACATCTGCTCAAACTCGAAGTC	8
TaqMan MGB Probe:	VIC-TCGATCTGGACATGTTGG-MGB	9

We found that O513 raised on 100 μg/ml tetracycline had a tTA:18S ratio of 0.00016 (larvae) and 0.00013 (adult). Adults raised as larvae on 100 μg/ml tetracycline, but then transferred to non-tetracycline diet as adults had ratios of 0.0061, 0.0047, 0.0087 and 0.011 after 1, 2, 4 and 8 days without tetracycline, respectively. This 28- to 64-fold increase in expression relative to the tetracycline-fed control indicates that the tTAV-based positive feedback expression system is functional in adults.

EXAMPLE 6

LA513 in *Aedes aegypti*

Aedes aegypti (the yellow fever mosquito, also the major vector of urban dengue fever) were transformed with LA513. Two independent insertion lines, LA513A and LA513B, were obtained.

Males heterozygous for LA513A (reared as larvae on 30 μg/ml tetracycline) were allowed to mate with wild type females. Eggs were collected and the resulting larvae raised in normal media, or in media supplemented with tetracycline (Tc) to 30 μg/ml. The number of transgenic and non-transgenic adults resulting from these eggs was determined. Data are the sum of at least 5 experiments. Larvae were reared at a density of ≤ 250 individuals per litre; all the eggs in "no tetracycline" experiments were washed twice before submergence to avoid transferring tetracycline. For the "with tetracycline" experiments, the parental blood and sugar-water was supplemented with tetracycline to 30 μg/ml; for the "no tetracycline" experiments it was not. χ^2 test for differentiation in ratio of the transgene and wild types for survival to adult: "with tetracycline", either orientation: $P>0.05$; "without tetracycline, either orientation $P<0.001$ (null hypothesis: genotype with respect to LA513 has no effect on survival).

LA513A is, therefore, a repressible dominant lethal, with a penetrance in these experiments of 95-97%. LA513B is also a repressible dominant lethal, with a penetrance in these experiments of 100%. The results are shown in Table 10, below.

Table 10

Effect of tetracycline on the survival of LA513/+ *Aedes aegypti* and their +/+ siblings.

Parents		Progeny								
Male	Female		Tc as larvae	Genotype						
		Egg			1st instar larvae	2nd	3rd	4th	Pupae	Adults
LA513A/+	+/+	1000	Yes	LA513A/+	489	468	446	442	437	434
				Wild type	444	431	403	400	396	392
+/+	LA513A/+	1000	Yes	LA513A/+	442	420	404	399	393	383
				Wild type	466	444	428	417	412	404
LA513A/+	+/+	540	No	LA513A/+	274	265	235	208	155	7
				Wild type	233	225	214	212	209	206
+/+	LA513A/+	497	No	LA513A/+	216	205	181	168	131	9
				Wild type	241	225	216	214	211	207

Parents		Progeny								
Male	Female		Tc as larvae	Genotype						
		Egg			1st instar larvae	2nd	3rd	4th	Pupae	Adults
LA513B/+	+/+	377	Yes	LA513B/+	161	153	147	141	139	131
				Wild type	178	171	165	160	157	153
+/+	LA513B/+	442	Yes	LA513B/+	189	181	170	166	161	153
				Wild type	203	198	185	182	180	176
LA513B/+	+/+	188	No	LA513B/+	69	19	0	0	0	0
				Wild type	85	84	83	83	82	81
+/+	LA513B/+	240	No	LA513B/+	91	60	0	0	0	0
				Wild type	107	104	99	98	95	93

We examined the survival of LA513A/+ males that had been raised on tetracycline (30μg/ml), as larvae, but not given tetracycline as adults. We found that all males tested survived for three weeks, irrespective of genotype (LA513A/LA513A, LA513A/+ or +/+) or the presence or absence of tetracycline in their diet (n≥40 for each genotype).

We examined the survival of LA513A/+ males that had been raised on tetracycline (30μg/ml), as larvae, but not given tetracycline as adults. We found that all males tested survived for three weeks, irrespective of genotype (LA513A/LA513A, LA513A/+ or +/+) or the presence or absence of tetracycline in their diet (n≥40 for each genotype).

We investigated the induction kinetics of tTAV in adult LA513B/+ mosquitoes after withdrawal of tetracycline, using qPCR. As shown in Table 11, below, tTAV increased in males and females following withdrawal of tetracycline. Induction of tTA expression is fairly rapid after removal of Tc, as with *Drosophila*. In each case, shifting between diets containing different levels of tetracycline provides a level of control over the expression level of genes controlled by tTA (here exemplified by tTA itself), using such a positive feedback system.

Table 11

Induction of tTA expression in LA513B/+ males following withdrawal of tetracycline

Sex	Time (days) without tetracycline	tTA:18S expression ratio	tTA:18S expression relative to male with tetracycline
Male	0	0.00036	1
Female	0	0.00060	1.7
Male	3	0.0043	12
Female	3	0.014	38
Male	4	0.054	150
Female	4	0.019	530
Male	8	0.012	34
Female	8	0.52	1500
Male	16	0.10	280
Female	16	0.032	88

EXAMPLE 7

Tetracycline-repressible enhancement of a nearby promoter by tTAV in a positive feedback configuration

We observed that the fluorescent marker in LA513A and LA513B transgenic mosquitoes showed a different pattern of fluorescence in the absence of tetracycline, compared with the pattern in the presence of tetracycline. Fluorescence in the presence of tetracycline was typical of Actin5C-driven expression in mosquitoes (Catteruccia *et al.*, 2000; Pinkerton *et al.*, 2000), and limited largely to the swollen part of the thorax. In contrast, in the absence of tetracycline, expression was much stronger and evident substantially throughout the body of transgenic individuals. In each case, assessment of fluorescence intensity and expression pattern was made by visual observation using fluorescence microscopy.

Elevated expression of tTAV in this positive feedback situation appears, therefore, to be stimulating expression from the nearby Actin5C promoter. This is illustrated, diagrammatically, in Figure 9. We also found that intermediate concentrations of tetracycline, just sufficient substantially to suppress the lethality of LA513, did not suppress this broader expression pattern of fluorescence. At these intermediate concentrations of tetracycline, tTAV accumulates to an intermediate level – sub-lethal, but higher than in 30 μg/ml tetracycline, and which still influences the expression of DsRed2. This again exemplifies the additional control available by modulating tetracycline concentration.

Figure 9 illustrates the bidirectional action of $tetO_7$ in 513A and 513B mosquitoes. In 513, DsRed2 is under the transcriptional control of the *Drosophila* Actin5C promoter.
(A) In the presence of tetracycline, relatively little tTAV is produced, this binds tetracycline and has little or no effect on DsRed2 expression. DsRed2 is seen in a pattern typical of Actin5C expression in mosquitoes.
(B) In the absence of tetracycline, tTAV stimulates its own expression in a positive feedback loop.
(C) tTAV binding to the tetO sites enhances expression of both the hsp70 minimal promoter, and hence tTAV, but also the Actin5C promoter, and hence DsRed2.

EXAMPLE 8

LA656, LA928 and LA1124 in *Ceratitis capitata*

No transgenic lines of the Mediterranean fruit fly (medfly, *Ceratitis capitata* Wiedmann) were obtained, using pLA513, probably indicating that the Actin5C-based marker of pLA513 is inappropriate for use in medfly. This emphasises the desirability of expression constructs with a wide species range. We, therefore, modified the construct to include a polyubiquitin (ubi-p63E)-based marker instead of the Actin5C-based one of pLA513. One such construct is pLA656. We also made two additional constructs, pLA928 and pLA1124 (SEQ ID NO's 18, 20 and 21, respectively, and shown, diagrammatically, in Figures 10, 11 and 12), using a marker based on the hr5 enhancer and ie1 promoter from a baculovirus (*Autographica californica* nuclear polyhedrosis virus, *Ac*MNPV). These differ in the orientation of the marker with respect to the tetO-tTAV cassette. The hr enhancer is closer to the tetO-tTAV cassette in pLA1124 than in pLA928. Furthermore, pLA1124 has 21, rather than 7, copies of tetO and additionally has a putative GAGA-factor binding region related to that of pUASp (Rorth, 1998).

One transgenic line was obtained from pLA656, three for pLA928, and three for pLA1124. These lines are assumed to have independent insertions, as they were derived from different G0 injection survivors.

Males heterozygous for each line were crossed to wild type females. The progeny were raised on standard yeast/sugar/wheatgerm or yeast/sugar/maize *Drosophila* diet, supplemented with tetracycline as appropriate. The parents were raised on the same diet, supplemented with tetracycline to 100μg/ml in the case of the transgenic males. The wild type females to which these males were mated were raised without tetracycline, to eliminate any potential maternal contribution of tetracycline. The number of transgenic and non-transgenic pupae and adults obtained from each cross was determined by scoring for DsRed2 by fluorescence microscopy.

The results of these crosses are shown in Table 12, below. In each case, in the absence of tetracycline, survival of the heterozygous transgenics was less than 2% relative to their wild type

shifting between diets containing different levels of tetracycline, modifying the construct, and using position effect, are discussed elsewhere herein.

Table 12

Effect of tetracycline on the survival of transgenic medfly heterozygous for various constructs, and their +/+ siblings

LA656	**Progeny [Tc] (µg / ml)**	**F/NF pupae**	**Pupal survival ratio (%)**	**F male**	**F female**	**NF male**	**NF female**	**Adult survival ratio (%)**
	0	84 / 1161	7	6	2	530	551	**0.7**
	0.1	16 / 423	4	0	0	205	177	**0**
	1	124 / 384	32	34	12	155	174	**14**
	3	258 /370	70	84	53	165	133	**46**
	10	249 / 252	99	91	98	107	127	**81**
	100	330 / 307	107	151	150	134	148	**107**

LA928m1	Progeny [Tc] (μg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	28 / 1499	1.87	5	1	661	639	**0.46**
	0.1	0 / 765	0	0	0	347	246	**0**
	1	190 / 256	74	62	59	119	101	**55**
	3	290 / 302	96	133	98	143	107	**92**
	10	nd	nd	nd	nd	nd	nd	**nd**
	100	222 / 286	77	117	84	146	126	**74**

LA928m3	Progeny [Tc] (μg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	68 / 1026	6.6	13	4	489	449	**1.8**
	0.1	0 / 265	0	0	0	117	91	**0**
	1	358 / 446	80	154	100	228	164	**65**
	3	105 / 105	100	39	35	42	38	**93**
	10	nd	nd	nd	nd	nd	nd	**nd**
	100	245 / 245	100	109	121	117	108	**100**

LA928f1	Progeny [Tc] (μg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	17 / 1331	1.3	2	0	639	599	**0.16**
	0.1	2 / 254	0.8	0	0	100	84	**0**
	1	461 / 567	81	218	146	244	181	**85**
	3	520 / 527	99	214	182	249	202	**88**
	10	350 / 399	91	139	112	131	159	**87**
	100	126 / 117	108	63	57	57	49	**113**

LA1124f1	Progeny [Tc] (μg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	104 / 213	51	0	3	95	62	**1.9**
	100	478 / 536	89	218	208	205	203	**104**

LA1124m1	Progeny [Tc] (μg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	337 / 437	77	2	1	176	207	**0.78**
	100	84 / 90	93	35	31	30	26	**118**

LA1124m2	Progeny [Tc] (µg / ml)	F/NF pupae	Pupal survival ratio (%)	F male	F female	NF male	NF female	Adult survival ratio (%)
	0	104 / 145	72	0	1	46	34	**1.3**
	100	77 / 77	100	24	14	19	13	**119**

F: fluorescent;

NF: non-fluorescent.

Pupae were collected and scored for fluorescence (column 3), then allowed to eclose. Surviving adults were scored for sex and fluorescence (columns 5-8). From these data on adults, the ratio of fluorescent to non-fluorescent survivors was calculated, presented in column 9 as the percentage of fluorescent adults observed relative to non-fluorescent. It is to be expected that these crosses give, on average, equal numbers of transgenic and non-transgenic individuals; if an equal proportion of transgenic and non-transgenic individuals were to survive to adulthood, then this would give an "adult survival ratio" of 100%.

We further investigated the expression of tTA in these transgenic lines by quantitative (real-time) rt-PCR (qPCR). The results are given in Table 13, below.

Table 13

Expression levels of tTA in wild type and transgenic medfly

Sample	tTA/18S ratio	NT/T ratio
Larvae		
WT tet	3.13E-06	
WT NT	2.81E-06	
656 tet	5.80E-06	1.00
656 NT	2.06E-04	36
670A tet	2.71E-06	1.00
670A NT	1.10E-04	41
670e tet	9.70E-06	1.00

670e NT	8.40E-05	8.7
Adults		
WT female	2.83E-06	
WT male	2.16E-07	
Heterozygous		
656 tet M 0d	5.52E-06	1.00
656 tet M 8d	1.12E-05	2.0
656 NT M 0d	4.49E-05	8.1
656 NT M 2d	2.77E-04	50
656 NT M 4d	2.22E-04	40
656 NT M 8d	9.71E-05	18
656 NT M 16d	1.49E-04	27
670 M tet	4.21E-06	1.00
670 F tet	2.86E-06	0.68
670 M NT S	6.93E-05	16.45
670 F NT S	1.92E-04	45.57
928Am1 F tet	7.17E-06	1.00
928Am1 M tet	8.56E-06	1.19
928Am1 M NT 2d	1.71E-04	23.81
928Am1 M NT 4d	5.36E-04	74.72
928Am1 M NT 8d	1.91E-04	26.66
928Am1 M NT 16d	1.01E-05	1.41
928Am1 M tet 8d	1.11E-06	0.16
928Am1 M NT S	2.22E-04	31.02
928Am1 M NT S	1.51E-04	21.11
928Am3 F tet	9.09E-07	1.00

928Am3 M tet	9.09E-07	1.00
928Am3 F NT S	3.62E-05	39.85
928Am3 F NT S	8.74E-04	962.07
928Am3 F NT S	2.99E-04	329.32
928Am3 M NT S	5.53E-05	60.83
928Am3 M NT S	9.18E-04	1009.90
1124f1 F tet	2.86E-05	1.00
1124f1 F NT 7d	4.11E-04	14.35
1124m1 M tet	1.62E-05	1.00
1124m1 F NT S	9.30E-04	57.55
1124m2 F tet	8.98E-05	1.00
1124m2 F NT 7d	7.90E-04	8.79
homozygous		
656 tet 8d	1.49E-05	1.00
656 NT 0d	9.23E-05	6.2
656 NT 2d	3.90E-03	262
656 NT 4d	1.92E-03	129
656 NT 8d	4.70E-03	316
656 NT 16d	8.58E-04	58

M: male;

F: female;

tet: raised on diet supplemented with tetracycline to 100 μg/ml;

NT S: raised on standard diet (0 μg/ml tetracycline);

d: days post-eclosion;

NT (n)d: raised on tet diet, then held as adults on non-tet (NT) diet for n days, as indicated;

tet (n)d: raised on tet diet, then held as adults on tet diet for n days, as indicated.

EXAMPLE 9

LA670 in *Ceratitis capitata*

We obtained a single transgenic line of medfly by transformation with pLA670, a construct which closely resembles pLA656. This plasmid is illustrated in accompanying Figure 13, and is SEQ ID NO. 23.

However, this transgenic line gave a significant number of adult transgenic progeny, even when raised as larvae on diet lacking tetracycline (Table 14). However, this LA670 insertion line does produce a readily detectable amount of tTAV mRNA in the absence of tetracycline, and this is substantially reduced by dietary tetracycline (assessed by qPCR, results shown in Table 13, above). LA670, therefore, represents a useful regulatable source of tTAV with which to drive the expression of tTAV-responsive genes. The difference in phenotype between LA656 and LA670, which are extremely similar in structure, is probably due to position effect, which is the variation in expression of transgenes depending on where they have inserted in the genome. Such variation is also shown by the variation in phenotype and tTAV expression levels between different transgenic lines with the same construct, as shown in Table 13, above. A simple method for obtaining transgenic lines carrying positive feedback constructs with different expression levels and phenotypic consequences is therefore provided, comprising generating a panel of insertion lines and screening for suitable basal and de-repressed expression levels and patterns.

Table 14

Effect of tetracycline on the survival of transgenic medfly heterozygous for LA670, and their +/+ siblings

LA670	**Progeny [Tc] (μg / ml)**	**F/NF pupae**	**Pupal survival ratio (%)**	**F male**	**F female**	**NF male**	**NF female**	**Adult survival ratio (%)**
	0	182 / 220	83	72	35	102	103	**52**
	100	10 / 8	125	5	3	5	3	**100**

F: fluorescent;

NF: non-fluorescent.

Pupae were collected and scored for fluorescence (column 3), then allowed to eclose. Surviving adults were scored for sex and fluorescence (columns 5-8). From these data on adults, the ratio of fluorescent to non-fluorescent survivors was calculated, presented in column 9 as the percentage of fluorescent adults observed relative to non-fluorescent. It is to be expected that these crosses give, on average, equal numbers of transgenic and non-transgenic individuals; if an equal proportion of transgenic and non-transgenic individuals survived to adulthood, this would give an "adult survival ratio" of 100%.

We tested the ability of LA670 to drive expression of sequences placed under the transcriptional control of tetO. We analysed the expression of two potential mRNAs from pLA1038 (Figure 14, SEQ ID NO. 24), which contains two potential tTA-responsive transcription units, divergently transcribed. These are CMV-tTA and hsp70-Cctra-nipper. PCR analysis, with controls, was performed on the expression of these transcription units in the presence and absence of pLA670. Both transcription units are expressed in the presence of pLA670. CMV-tTA is expressed at a lower, but detectable, level in LA1038/+ transgenics in the absence of LA670. hsp70-Cctra-nipper is not detectably expressed in the absence of pLA670, showing that expression is indeed driven by, and dependent on, tTAV supplied by pLA670.

EXAMPLE 10

LA710 in *Pectinophora gossypiella*

Pectinophora gossypiella (pink bollworm, a lepidopteran) was transformed with LA710 (Figure 15, SEQ ID NO. 19) by standard methods (Peloquin *et al.*, 2000). Four transgenic lines were recovered. Males of these lines were crossed with females wild type for LA710. Newly hatched larvae were placed in individual 1.7 ml vials with diet, either with or without 7-chlortetracycline (40μg/ml), and scored for fluorescence. No significant difference was observed in the numbers of transgenics surviving to adulthood relative to numbers of their wild type siblings, either with or without chlortetracycline. We conclude that LA710 does not typically lead to the accumulation of lethal levels of tTAV, even in the absence of dietary chlortetracycline.

We examined the expression of tTAV mRNA in LA710 transgenics by PCR following a reverse transcriptase reaction (rt-PCR). We found that tTAV mRNA was not detectable in chlortetracycline-fed larvae, but was detectable in larvae which had not received chlortetracycline (data not shown). This positive feedback construct LA710, therefore, provides, in these moths, a source of tTAV that can be regulated by supplying dietary chlortetracycline, and for which de-repressed expression, though readily detectable, is non-lethal. We also observed significant variation in the intensity of the band corresponding to tTAV mRNA in samples from different lines.

EXAMPLE 11

LA1124 in *Pectinophora gossypiella*

Pectinophora gossypiella (pink bollworm, a lepidopteran) was transformed with LA1124 (Figure 12, SEQ ID NO. 21) by standard methods (Peloquin *et al.*, 2000). A single transgenic line was recovered. Males of this line were crossed with females wild type for LA1124. Newly hatched larvae were placed in individual 1.7 ml vials with diet, either with or without 7-chlortetracycline (40μg/ml), and scored for fluorescence. These larvae were screened again when they had had time to develop to a late larval stage. All larvae survived, except for the fluorescent (LA1124/+) larvae on diet lacking chlortetracycline, as shown in Table 15, below.

Table 15

Pink bollworm: survival from early to late larval stage of LA1124/+ or their wild type siblings, on diet with or without chlortetracycline

100 μg/ml chlortetracycline		**0 μg/ml chlortetracycline**	
LA1124/+	**Wild-type**	**LA1124/+**	**Wild-type**
3 (0 dead)	11 (0 dead)	8 (8 dead)	7 (0 dead)

We examined the expression of tTAV mRNA in LA1124 pink bollworm by PCR following a reverse transcriptase reaction (rt-PCR). We found that tTAV mRNA was readily detectable in chlortetracycline-fed larvae, but considerably elevated in larvae which had not received chlortetracycline (data not shown). The significant basal expression of tTAV mRNA in

this construct is probably due to the inclusion in LA1124 of the hr enhancer, which was included for this reason. Comparison of the structure and function of LA1124 with that of LA710 clearly illustrates that basal and maximum levels of the gene product can readily be selected by appropriate modification of the expression construct, this principle being demonstrated, here, by regulating levels of expression of a tTAV-dependent RNA (in this case the tTAV mRNA).

EXAMPLE 12

Sex-specific expression using positive feedback

It is preferred to control, by design, the expression of tTAV from a positive feedback construct, so that it can be differentially expressed in different tissues, or different developmental stages, or different sexes, for example. One application for this is in genetic sexing, in which a sexual dimorphism is induced between the two sexes and this is used as a basis for separating the two sexes. In the context of the Sterile Insect Technique, *e.g.* for medfly, this preferably means killing the females, most preferably at an early stage in their development. No early-acting female-specific promoters are known for medfly, which limits the potential of the two-component repressible dominant lethal system exemplified for *Drosophila* using promoters or enhancers from yolk protein genes (Heinrich and Scott, 2000; Thomas *et al.*, 2000). It would clearly be advantageous to be able to combine the beneficial characteristics of a conditional positive feedback system with a mechanism conferring female specificity.

We, therefore, modified a non-sex-specific positive feedback construct by inserting a sex-specific intronic region from *Cctra*, the medfly homologue of the *Drosophila melanogaster* gene *transformer* (Pane *et al.*, 2002). The sex-specific splicing of *Cctra* is illustrated diagrammatically in Figure 16, which is adapted from (Pane *et al.*, 2002)*supra*. Figure 16 shows the genomic organisation of the medfly *tra* gene. The top line represents the genomic *Cctra* locus. Exons are shown as blocks; aug marks the shared start codon. The alternate splice junctions are marked i. Putative tra/tra-2 binding sites are marked with arrowheads. Transcript F1, the only one to encode functional Cctra protein, is specific to females. Transcripts M1 and M2 are found in both males and females.

Three main transcripts are produced: M1, M2 and F1. Transcript F1 is found only in females, and is the only one to encode full-length, functional Cctra protein. Transcripts M1 and M2 are found in both males and females, and include additional exonic sequence, which inserts one or more stop codons relative to transcript F1, leading to truncation of the open reading frame.

We inserted the *Cctra* intron into the open reading frame of tTAV, so that excision by splicing of the complete intron, in the manner of transcript F1, would reconstitute an intact tTAV coding region, but splicing in the manner of either M1 or M2 would result in a truncated protein incapable of acting as a transcriptional enhancer. The resulting plasmid, pLA1188 (Figure 17, SEQ ID NO. 22), was injected into medfly embryos. Surviving larvae were recovered, and extracts from these larvae were analysed by rt-PCR to determine the splicing pattern of the tTAV transcript.

Female larvae yielded PCR products corresponding to the expected sizes that would result from splicing in the pattern of the endogenous Cctra gene, in other words corresponding to splicing in the M1, M2 and F1 patterns. These data indicate that the *Cctra* intron can splice correctly in a heterologous context and, therefore, provides a suitable method for introducing sex-specificity into a positive feedback construct. Furthermore, since *tra* function is conserved across a wide phylogenetic range (Saccone *et al.*, 2002), and other sex-specific introns are known, *e.g.* in the *Drosophila melanogaster* gene *double-sex* (*dsx*), which is also well conserved, this provides a general method for manipulating the expression of genes. It will be apparent to the person skilled in the art that such manipulations can alternatively, or additionally, be applied to other genes responsive to a transcriptional activator, so that sex-specific expression of a target gene can be achieved by combining non-sex-specific expression of a transcriptional activator with sex-specific expression, *e.g.* through splicing, of a functional RNA under the transcriptional control of the transcriptional activator. Furthermore, it will also be apparent that this provides a simple mechanism for differential expression of two, or more, different target genes, or gene products, such that one, or one group, is expressed in both sexes and the other, or other group, in only one sex. This is illustrated for medfly in Figure 18.

The primers used were :
Tra(tTAV)Seq+: 5'-CCTGCCAGGACTCGCCTTCC (SEQ ID NO. 12)
Tra(tTAV)Seq-: 5'-GTCATCAACTCCGCGTTGGAGC (SEQ ID NO. 13)

RT-PCR products of ~600 and ~200 bp were produced when cDNA derived from female medflies 1 and 2 was used as a template, representing "male" (M1 and M2) and female-specific (F1) spliced forms of mRNA respectively (data not shown). The ~200 bp product could have been produced due to contamination with tTAV DNA – the female spliced form completely removes the Cctra intron an so leads to a PCR product that is identical to that which would be obtained from any of several tTAV-containing plasmids or samples handled in the same laboratory. The ~600 bp band, in contrast, retains ~400bp of Cctra sequence and is diagnostic of correct splicing of the construct.

In another experiment (data not shown), expression of transcripts from LA1038 in response to tTAV from LA670 was analysed by gel chromatography (data not shown), using:
A: rt-PCR for expression of CMV-tTA from LA1038 in extracts from LA1038/+, LA670/+ double heterozygotes;
B: rt-PCR for expression of hsp70-Cctra-nipper in extracts from LA1038/+, LA670/+ double heterozygotes; and
C: rt-PCR for expression of CMV-tTA from LA1038 in extracts from LA1038/+ heterozygotes without LA670.

All flies were raised in the absence of dietary tetracycline. In A and C, two bands were present between 200 bp and 400 bp and represent cDNA from spliced mRNA (lower molecular weight band) and genomic DNA or cDNA from unspliced message (higher molecular weight band) respectively. In B, a band at approximately 200 bp represents cDNA from mRNA spliced in the pattern of the Cctra female-specific F1 transcript, an upper band of approximately 1500bp representing genomic DNA or cDNA from unspliced message, and bands of intermediate size representing cDNA spliced in the pattern of the Cctra non-sex-specific M1 and M2 transcripts, or non-specific bands.

Primer sequences used were:

hsp70-Cctra-nipper: NIP: 5'-CATCGATGCCCAGCATTGAGATG and
HSP: 5'-CAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTA;
CMV-tTA: CMV: 5'- GCCATCCACGCTGTTTTGACCTCCATAG and
TTA: 5'-GCCAATACAATGTAGGCTGCTCTACAC

These data (not shown) demonstrate that the hsp-Cctra-nipper section of LA1038 is shown to be correctly spliced in the female form in 6/6 females, and in the male form in 6/6 males.

Reference sequences:

JY2004-tTA (SEQ ID NO. 14) - sequence of the tetO_7-tTA region only

pP[Casper-Act5C-tTA] (SEQ ID NO. 15)

pLA513 (SEQ ID NO. 16)

pLA517 (SEQ ID NO. 17)

pLA656 (SEQ ID NO. 18)

pLA670 (SEQ ID NO. 23)

pLA710 (SEQ ID NO. 19)

pLA928 (SEQ ID NO. 20)

pLA1038 (SEQ ID NO. 24)

pLA1124 (SEQ ID NO. 21)

pLA1188 (SEQ ID NO. 22)

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Scott, M., Heinrich, J., and Li, X. (2004). Progress towards the development of a transgenic strain of the Australian sheep blowfly (*Lucilia cuprina*) suitable for a male-only sterile release program. Insect Biochem Mol Biol *34*, 185-192.
Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. (1995). A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. Proc Nat'l Acad Sci (USA) *92*, 6522-6526.
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Varshavsky, A. (2000). Ubiquitin fusion technique and its descendants. Meth Enz *327*.

Claims:

1. An insect gene expression system, comprising at least one gene to be expressed and at least one promoter therefor, wherein a product of a gene to be expressed serves as a positive transcriptional control factor for the at least one promoter, and whereby the product, or the expression of the product, is controllable.

2. A system according to claim 1, wherein an enhancer is associated with the promoter, the gene product serving to enhance activity of the promoter *via* the enhancer.

3. A system according to claim 2, wherein the control factor is the tTA gene product or an analogue thereof, and wherein one or more tetO operator units is operably linked with the promoter and is the enhancer, tTA or its analogue serving to enhance activity of the promoter *via* tetO.

4. A system according to claim 4, in which the gene encodes the tTAV or tTAF product.

5. A system according to any preceding claim, wherein the gene is modified to at least partially follow codon usage in a species in which the system is for use.

6. A system according to any preceding claim, wherein the promoter is substantially inactive in the absence of the positive transcriptional control factor.

7. A system according to any preceding claim, wherein the promoter is a minimal promoter.

8. A system according to claim 7, wherein the promoter is selected from: hsp70, a P minimal promoter, a CMV minimal promoter, an Act5C-based minimal promoter, a BmA3 promoter fragment, an Adh core promoter, and anAct5C minimal promoter, or combinations thereof.

9. A system according to any preceding claim, wherein the promoter is derived from, or is a fragment of, CMV or Hsp70.

10. A system according to any preceding claim which substantially reduces fitness when activated or de-repressed.

11. A system according to claim 10, comprising a lethal gene under the control of the a promoter of the system.

12. A system according to claim 11, wherein the lethal gene is a dominant lethal.

13. A system according to claim 11 or 12, wherein the lethal gene and the positive control are the same.

14. A system according to claim 13, wherein the gene is tTA or an analogue thereof.

15. A system according to claim 11 or 12, wherein the lethal gene and positive control gene are different.

16. A system according to claim 10, wherein the reduced fitness is a high mortality rate.

17. A system according to any preceding claim, wherein expression of the positive control gene is selective.

18. A system according to claim 17, wherein expression of the gene is determined by sex.

19. A system according to claim 18, comprising a *doublesex, transformer* or sex-specific lethal sequence.

20. A system according to any preceding claim, wherein an effector gene is operably linked with at least one said promoter.

21. A system according to claim 20, wherein the effector gene is a dominant lethal gene.

22. A system according to claim 20, wherein the effector gene encodes RNAi.

23. A system according to any of claims 20 to 22, wherein activation of a promoter to which the effector gene is operably linked leads to a selective effect *via* a transcription or translation product of DNA under the control of the promoter.

24. A system according to any of claims 17 to 23, wherein selection is species specific.

25. A system according to any of claims 17 to 24, wherein selection is developmental stage specific.

26. A system according to any preceding claim, which is at least one cistron.

27. A system according to claim 26, which is at least two cistrons, said cistrons being linked to an enhancer under the control of the positive control gene.

28. A system according to any preceding claim, wherein expression of the positive control gene on removal of a suppressor for the gene has substantially no effect on the fitness of an adult from which the suppressor has been removed.

29. A system according to any preceding claim, bounded by insulator elements.

30. A system according to claim 29, wherein the insulators are non-identical insulators.

31. pLA513 as identified by SEQ ID NO. 16.

32. JY2004-tTA as identified by SEQ ID NO. 14.

33. A vector comprising the system of any of claims 1 to 30.

34. A vector according to claim 33, further comprising an expression marker.

35. A vector according to claim 34, wherein the expression marker is a fluorescent protein or resistance marker.

36. A vector according to any of claims 33 to 35, further comprising an expressible transposase gene.

37. An insect comprising, in its genome, a system according to any of claims 1 to 30.

38. An insect according to claim 37, which is substantially uncompromised by the system under permissive conditions where the positive control gene is not expressed.

39. An insect according to claim 37 or 38 which is from a pest species.

40. An insect according to any of claims 37 to 39, which is selected from: mosquito, bollworm, medfly, and *Drosophila.*

41. An insect according to any of claims 37 to 40, wherein expression of the positive control gene is blockable or controllable by dietary supplements.

42. A method to establish compatibility of a promoter with a species, comprising transforming said species with a plasmid, or other vector, comprising a system according to any of claims 1 to 28 with the promoter to be tested, said promoter being operably associated with a gene to be assayed, said plasmid further comprising a marker, under the control of a promoter appropriate to said species, the method further comprising assaying putative transgenic individuals for expression of the marker, and wherein individuals expressing the marker are subsequently assayed for expression of the gene to be assayed.

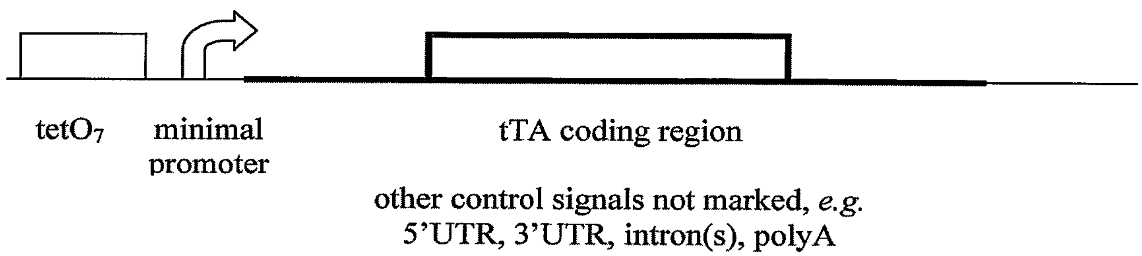

tetO7
minimal promoter
tTA coding region
other control signals not marked, e.g. 5'UTR, 3'UTR, intron(s), polyA

Fig.1

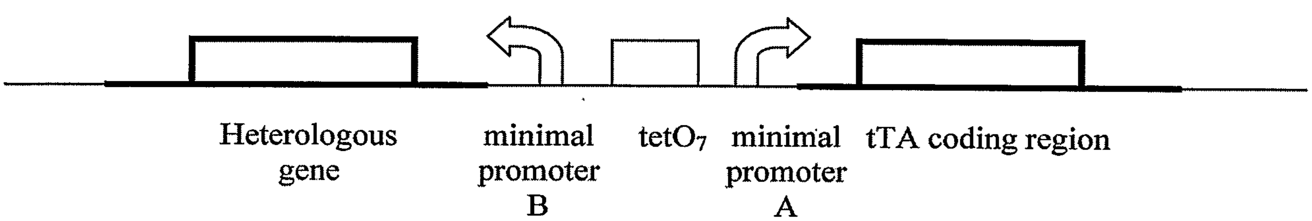
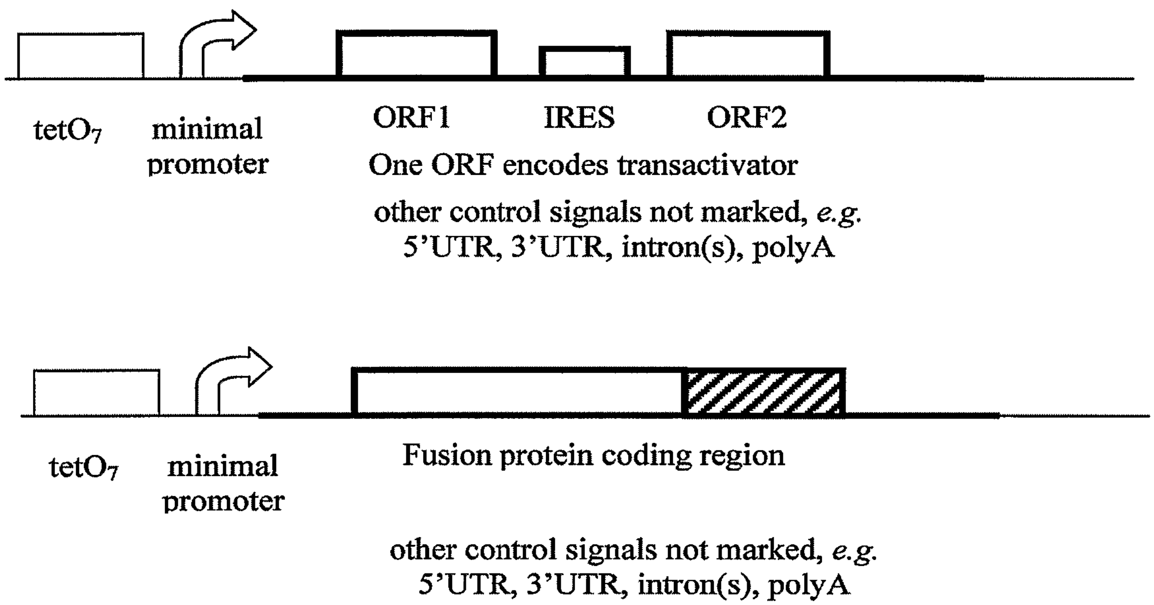
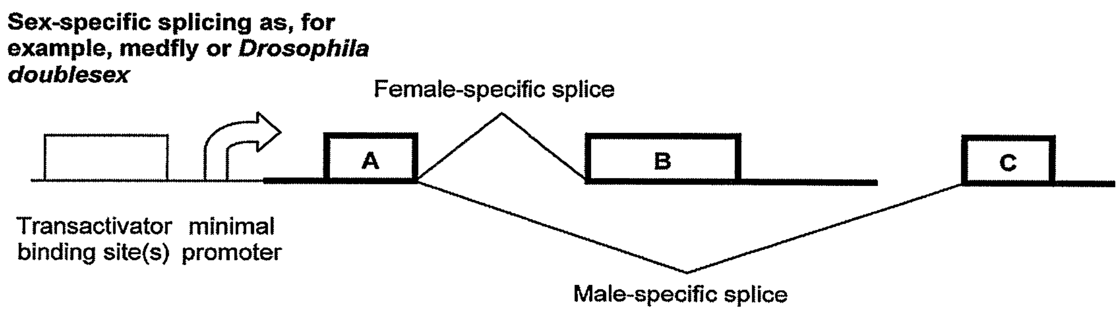

Heterologous gene
minimal promoter B
tetO7
minimal promoter A
tTA coding region

Fig.2

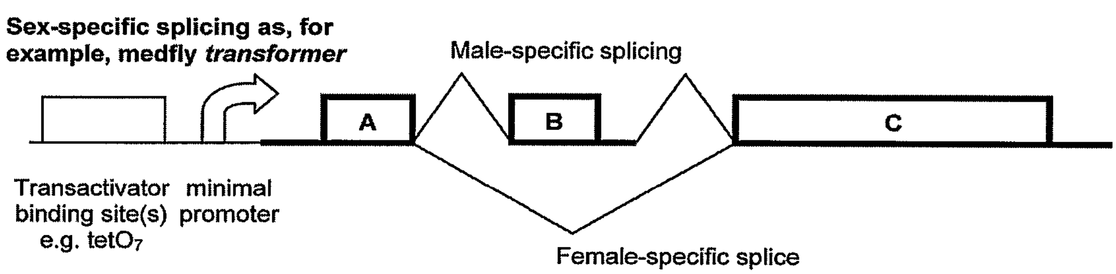


tetO$_7$
minimal promoter
ORF1
IRES
ORF2
One ORF encodes transactivator
other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA
tetO$_7$
minimal promoter
Fusion protein coding region
other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

Fig.3



Transactivator coding region:
A = DNA binding domain
B = Activation domain
C = Repression or neutral domain
Other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA



Transactivator coding region:
A + C = transactivator
B = contains stop codon or frame shift
or
A = DNA binding domain
B = Repression domain
C = Activation domain
Other control signals not marked, e.g. 5'UTR, 3'UTR, intron(s), polyA

Fig.4

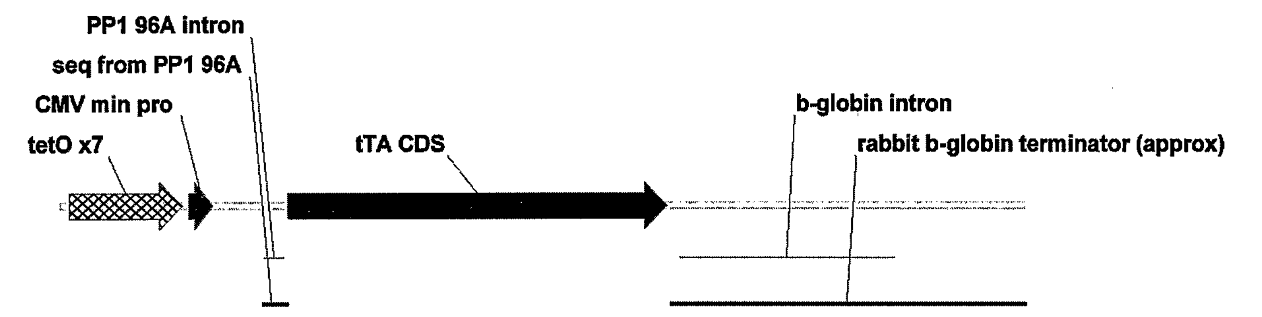


Diagram of tetO$_7$-tTA region of pJY2004

Fig.5

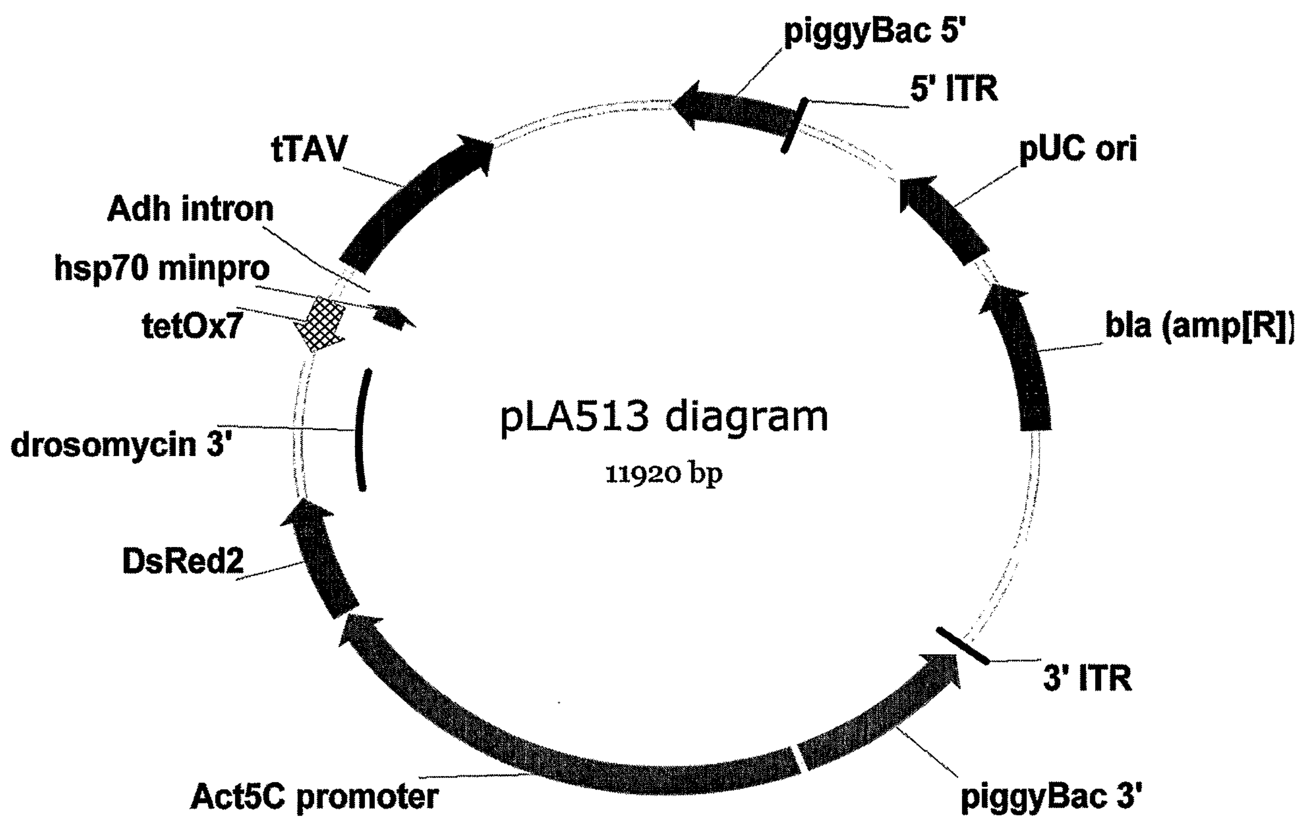
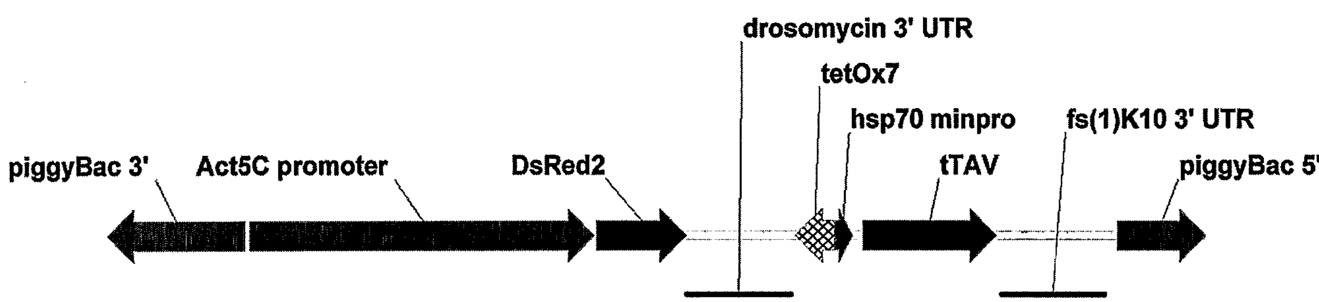
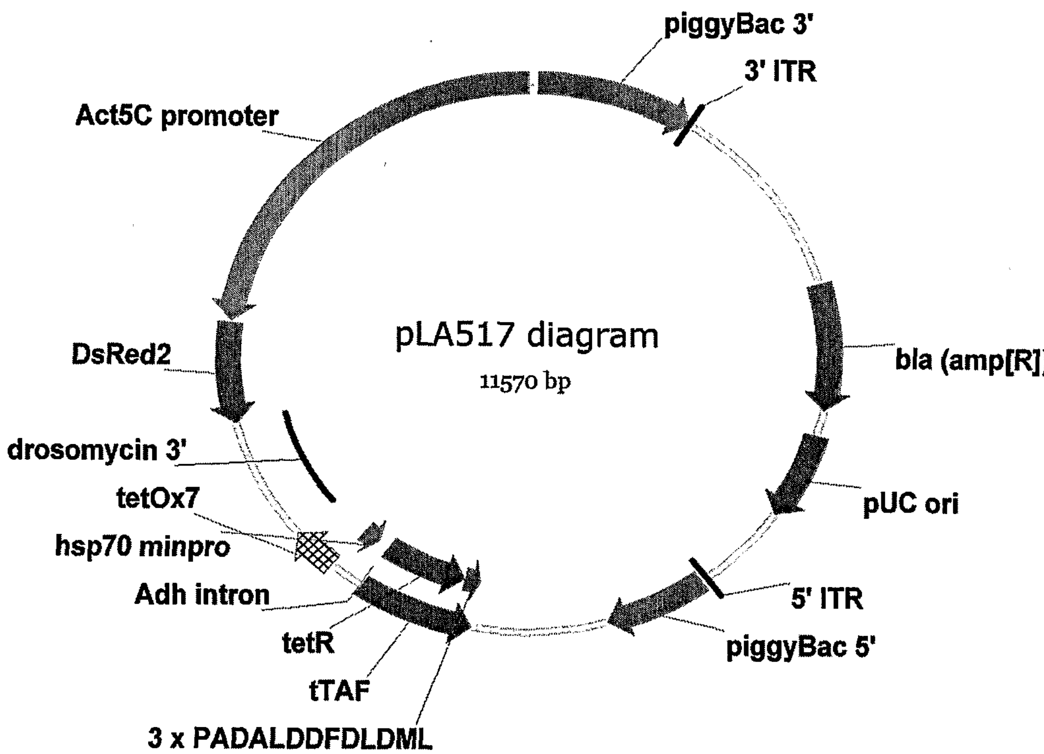


Fig.6



Schematic diagram of the LA513 transposon

Fig.7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3' ITR
Act5C promoter
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DsRed2
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drosomycin 3'
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pUC ori
Adh intron
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tetR
piggyBac 5'
tTAF
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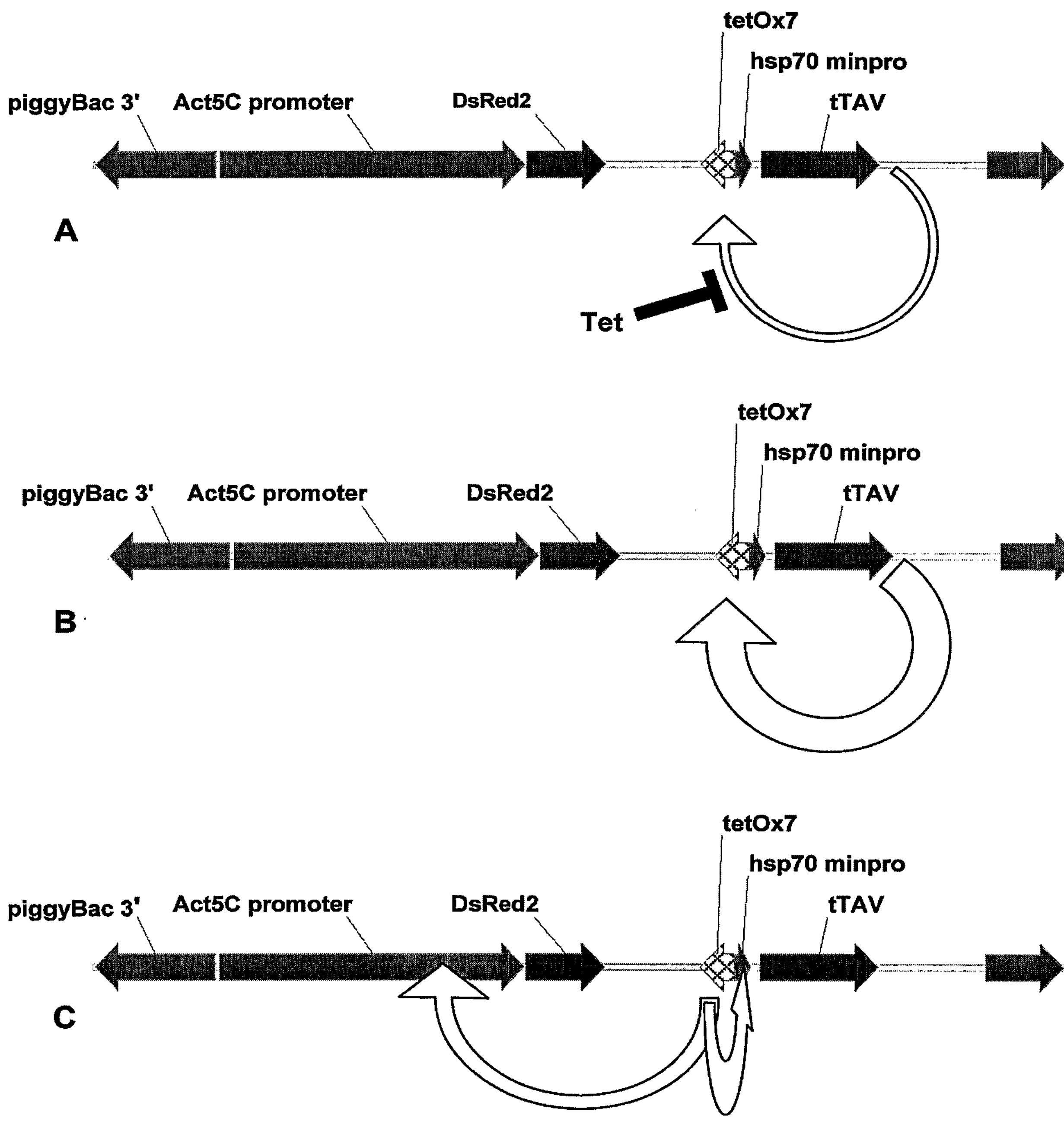
Fig.8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Act5C promoter
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tTAV
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Tet
tetOx7
hsp70 minpro
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Act5C promoter
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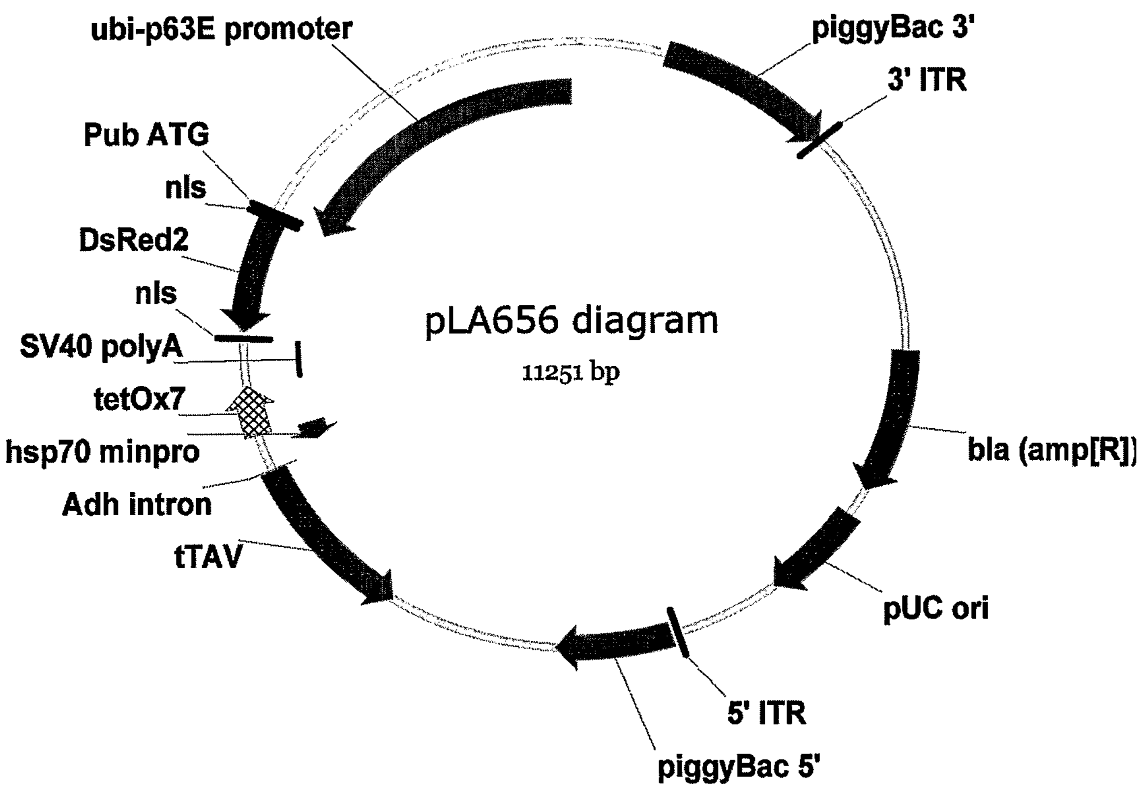
Act5C promoter
DsRed2
tTAV
C

Fig.9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nls
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tTAV
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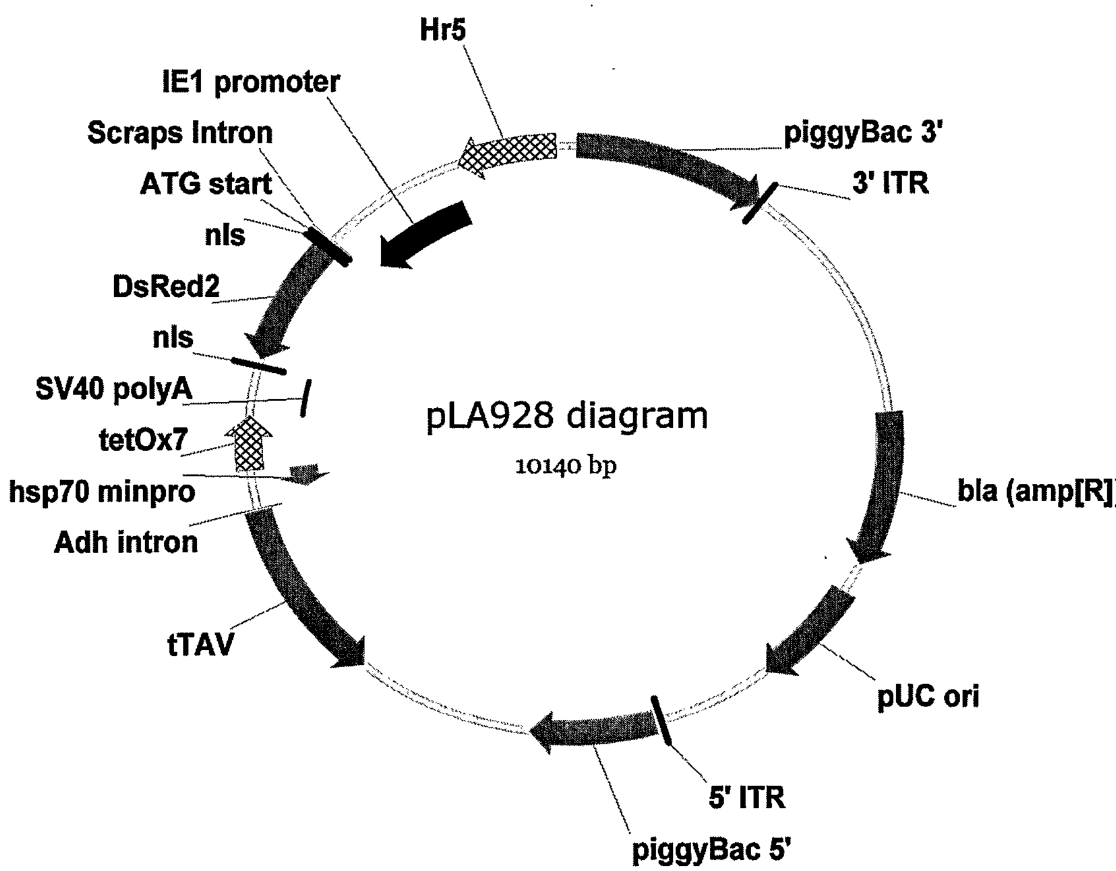
5' ITR
piggyBac 5'

Fig.10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Scraps Intron
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nls
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hsp70 minpro
Adh intron
tTAV
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5' ITR
piggyBac 5'
pLA928 diagram
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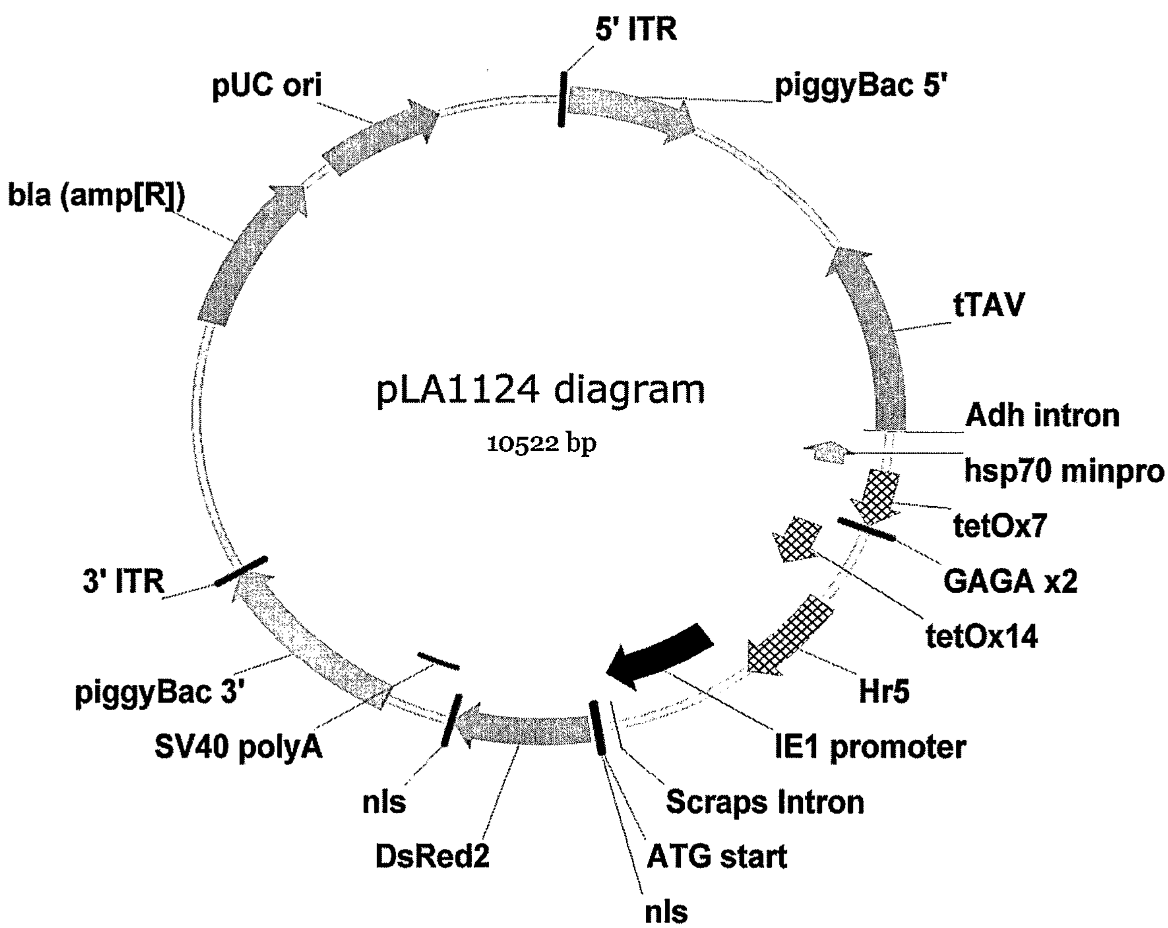
Fig.11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pUC ori
bla (amp[R])
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Adh intron
hsp70 minpro
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GAGA x2
3' ITR
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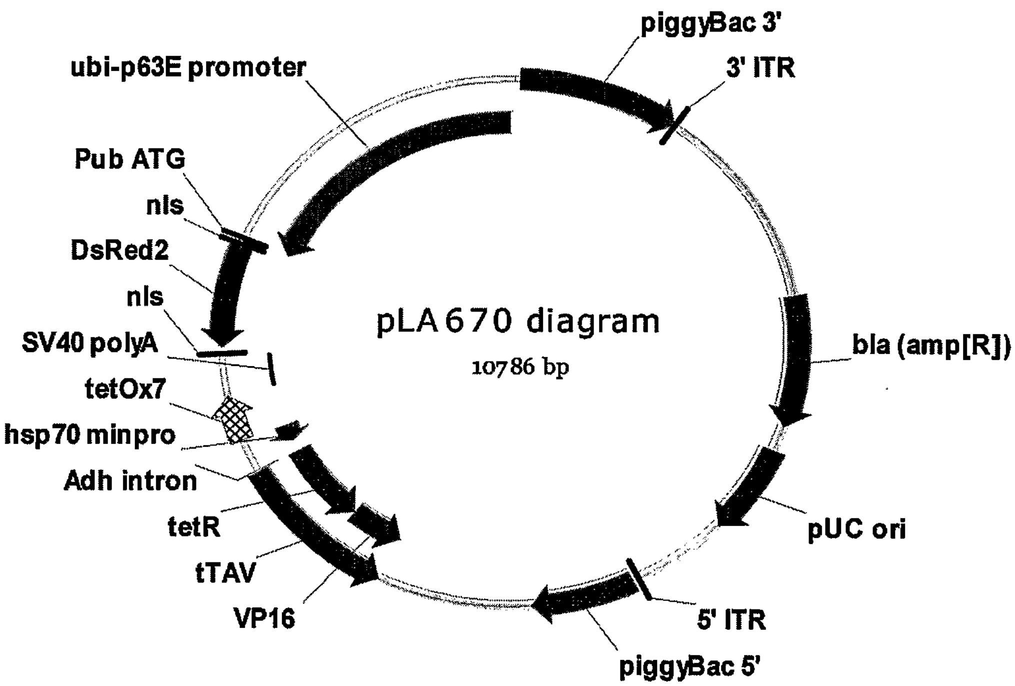
nls
Scraps Intron
DsRed2
ATG start
nls

Fig.12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tetR
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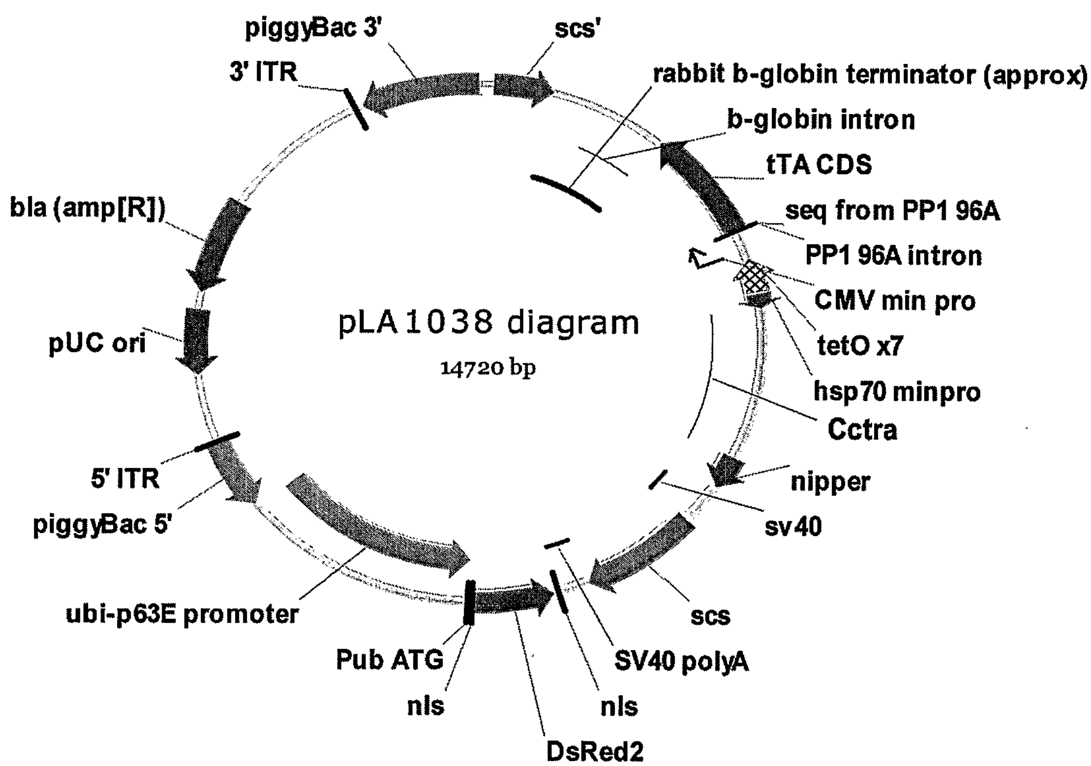
piggyBac 5'

Fig.13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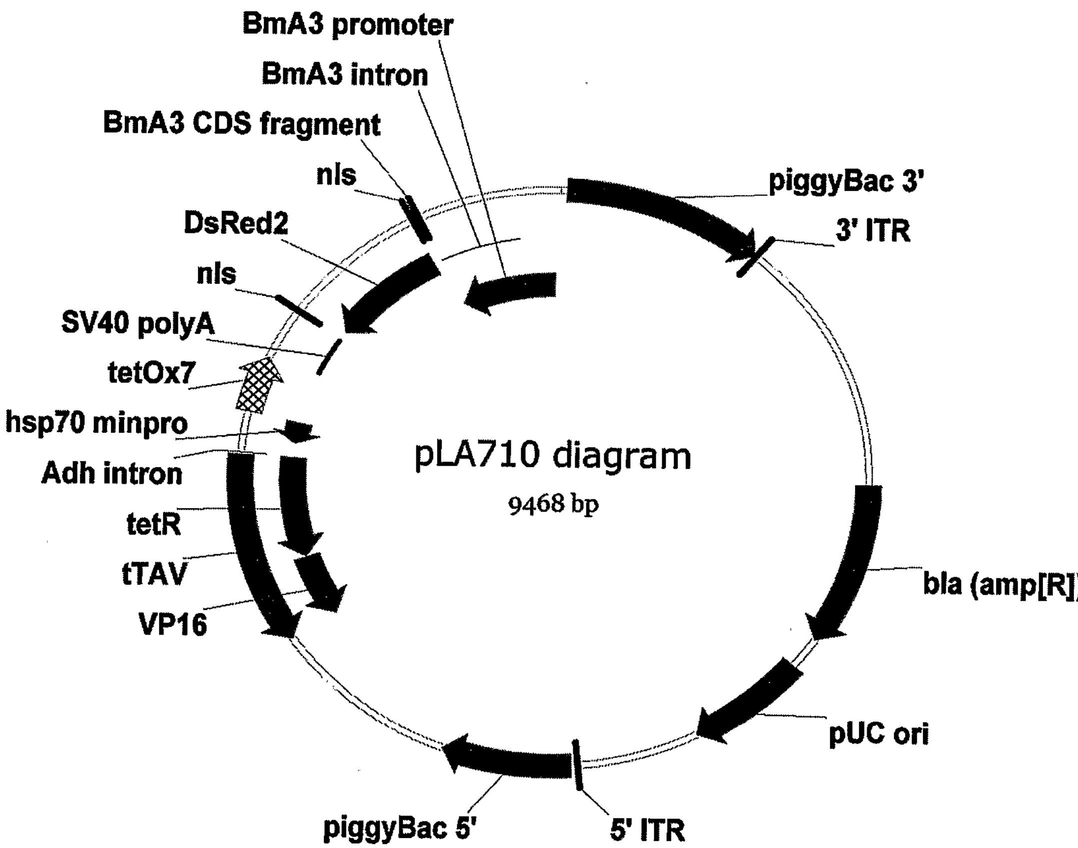
scs'
3' ITR
rabbit b-globin terminator (approx)
b-globin intron
tTA CDS
bla (amp[R])
seq from PP1 96A
PP1 96A intron
CMV min pro
pLA1038 diagram
14720 bp
pUC ori
tetO x7
hsp70 minpro
Cctra
nipper
5' ITR
sv40
piggyBac 5'
scs
ubi-p63E promoter
Pub ATG
SV40 polyA
nls
nls
DsRed2

Fig.14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BmA3 intron
BmA3 CDS fragment
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DsRed2
nls
SV40 polyA
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hsp70 minpro
Adh intron
tetR
tTAV
VP16
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3' ITR
pLA710 diagram
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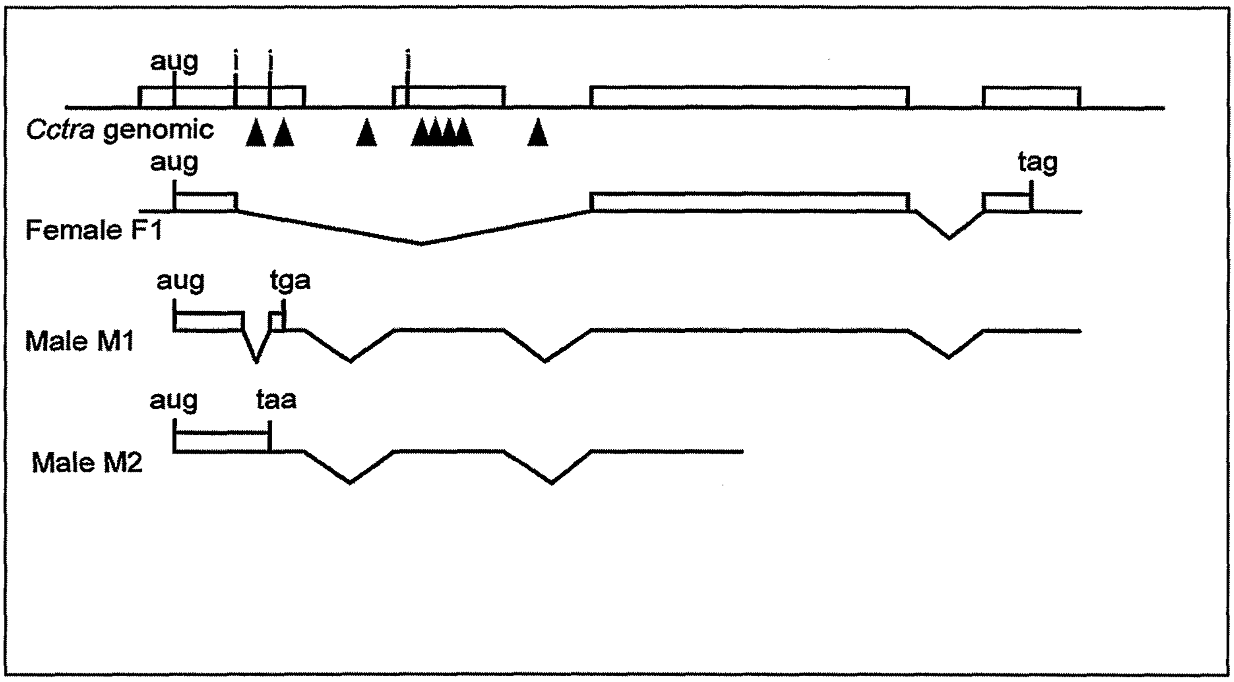
bla (amp[R])
pUC ori
piggyBac 5'
5' ITR

Fig.15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i
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i
Cctra genomic
aug
tag
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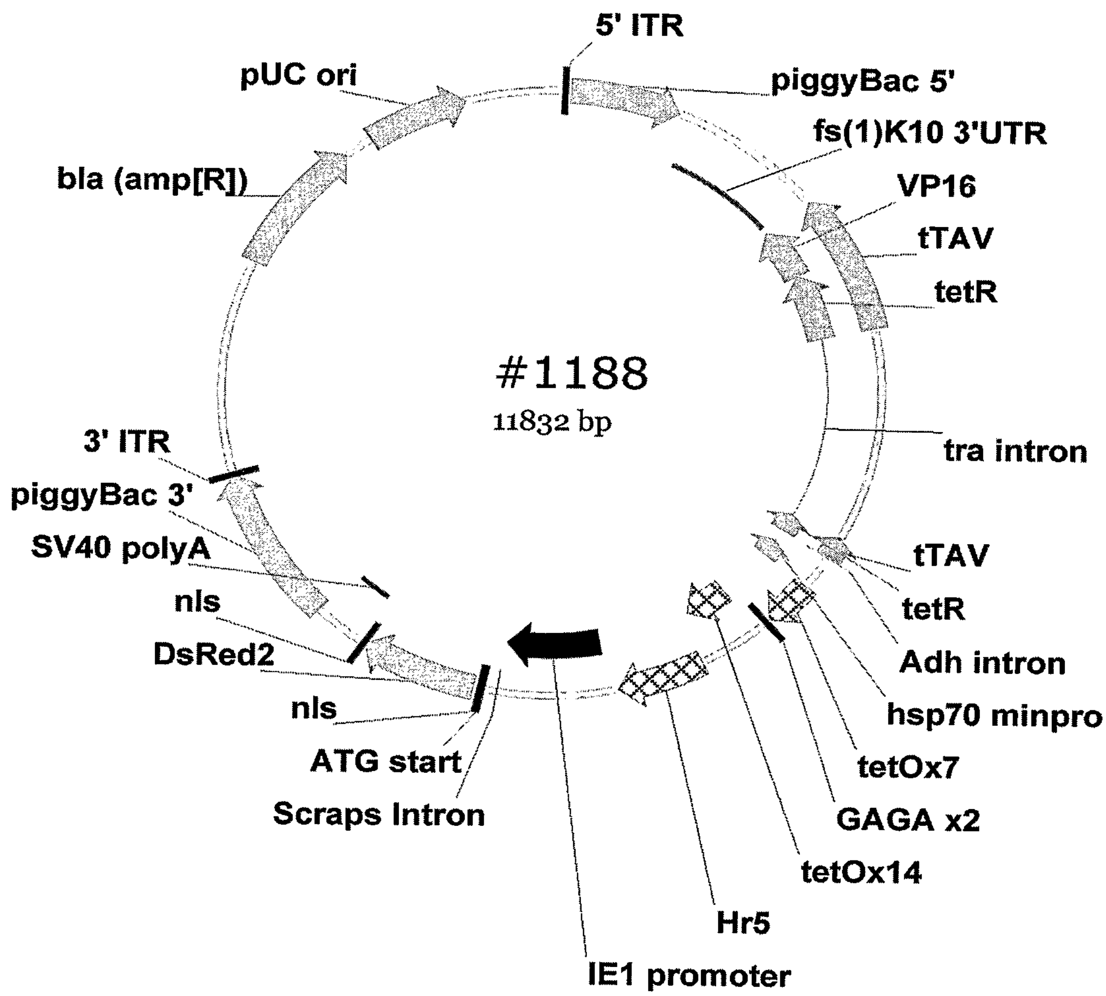
aug
tga
Male M1
aug
taa
Male M2

Fig.16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fs(1)K10 3'UTR
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VP16
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tetR
#1188
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tra intron
3' ITR
piggyBac 3'
SV40 polyA
tTAV
tetR
nls
Adh intron
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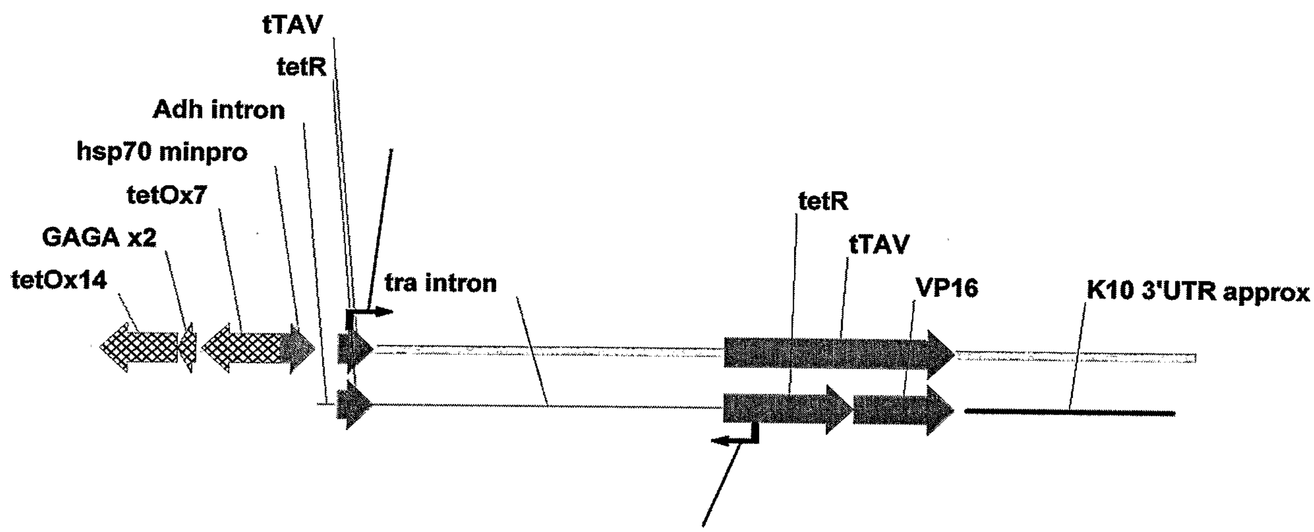
ATG start
Scraps Intron
GAGA x2
tetOx14
Hr5
IE1 promoter

Fig.17



Potential PCR products generated:

1. If intron is not excised → ~1550 bp
2. If intron is spliced in male form (M1 or M2)→ ~600 bp
3. If intron is spliced in female form → ~200 bp

Fig.18

SEQUENCE LISTING

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<150> UK 0317656.7
<151> 2003-07-28

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ctatcagtga tagagaaaag tgaaagtcga gtttaccact ccctatcagt gatagagaaa 180

agtgaaagtc gagtttacca ctccctatca gtgatagaga aaagtgaaag tcgagtttac 240

cactccctat cagtgataga gaaaagtgaa agtcgagttt accactccct atcagtgata 300

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Lys Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His
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His Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu
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Arg Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp
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Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu
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Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu
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Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly
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Cys Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu
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Leu Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu
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Leu Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser
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Ala Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser Thr Ile Glu
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Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu
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Asp Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe
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Asp Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr
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Gln Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn
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Lys Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His
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His Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu
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Arg Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp
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Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu
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Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu
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Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly
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Cys Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu
145 150 155 160

Thr Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu
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Leu Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu
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Leu Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser
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Gly Pro Ala Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser Thr
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Arg Leu Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu
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His Leu Asp Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp
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Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly
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cgaggccctt tcgtc 4455

INTERNATIONAL SEARCH REPORT

Application No
/GB2004/003263

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 A01K67/033

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HORN C ET AL: "A transgene-based, embryo-specific lethality system for insect pest management." NATURE BIOTECHNOLOGY, vol. 21, no. 1, January 2003 (2003-01), pages 64-70, XP002301699 ISSN: 1087-0156 the whole document -----	1-30, 33-42
X	WO 01/39599 A (ALPHEY LUKE ; THOMAS DEAN (GB); ISIS INNOVATION (GB)) 7 June 2001 (2001-06-07) the whole document -----	1-30, 33-42
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 October 2004	05/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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